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"Μεταλλαξιγένεση της *Lotus japonicus LSK*-1 χρησιμοποιώντας ένα σύστημα CRISPR/Cas9"

Μαγδαληνή Τσιτσικλή

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Ευχαριστίες

Η παρούσα μεταπτυχιακή διατριβή σημαίνει το τέλος των μεταπτυχιακών μου σπουδών στο τμήμα Βιοχημείας και Βιοτεχνολογίας.

Θα ήθελα να εκφράσω τις θερμότερες ευχαριστίες μου στους ανθρώπους που αφιέρωσαν πολύτιμο χρόνο, και έδειξαν μεγάλη υπομονή και επιμονή, ώστε να μπορέσει να υλοποιηθεί με τις καλύτερες προδιαγραφές αυτό το μεταπτυχιακό πρόγραμμα. Αναφέρομαι στους καθηγητές μου, κ Ματθιόπουλο Κωνσταντίνο, κα Παπαδοπούλου Καλλιόπη, κα Ψαρρά Άννα-Μαρία και κ Γιακουντή Αντώνη.

Η μεταπτυχιακή μου διατριβή, είναι άλλος ένας λόγος για τον οποίο θα ήθελα να ευχαριστήσω την κα Παπαδοπούλου, διότι μου έδωσε την ευκαιρία να γίνω μέλος της ερευνητικής της ομάδας, ενώ ήταν δίπλα μου και με συμβούλευε από την αρχή μέχρι το τέλος της. Η ερευνητική αυτή ομάδα έγινε τελικά μια δεύτερη οικογένειά για μένα, στην οποία χρωστάω ευγνωμοσύνη.

Last but not least, θα ήθελα να αφιερώσω την παρούσα διατριβή στον μέντορά μου, Κωνσταντίνο Γκαραγκούνη, και να τον ευχαριστήσω ιδιαίτερα για όλα αυτά που έχει κάνει για μένα κατά την διάρκεια των σπουδών μου.

Contents

Abstract

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems are utilized by archaea and bacteria as an adaptive immune system that recognises and disrupts foreign DNA. Currently, with some modifications, these systems are useful tools for researchers, since they provide the capability of precise genome editing in a wide range of species. Here, we report a methodology for the optimization of such a tool based on the TypeII CRISPR/Cas system. More specifically, we focus on the *in vitro* construction of CRISPR/Cas9 system components, compatible with the *Lotus japonicus* model plant and closely related legume species. This will lead to an increase of the transgene's expression levels and subsequently higher efficiency of genome editing because of both quantitative and qualitative alternations in the transgenes' expression. In the current work, we describe the construction of a gRNA expression cassette that consists of the *Lotus japonicus* RNApolIII-dependent U6 promoter, followed by a universal gRNA cloning site fused with the gRNAscaffold. Along with that, another binary plasmid vector was utilised in order to test our system. This last vector contains a Cas9 expression cassette consisting of a human optimised Cas9 nuclease driven by the Cauliflower Mosaic Virus 35S Promoter, widely used by many scientific groups for transgene expression in various plant species. Finally, to validate our constructs, we co-transformed *Lotus japonicus* roots via *Agrobacterium rhizogenes*-mediated hairy root transformation, with both the Cas9 cassette and the gRNA cassette. This particular guide RNA will recruit the Cas9 nuclease at the 11th exon of the *LSK1* gene for targeted mutagenesis. By further studying the resulting plant lines, we will have the opportunity to determine the role and function of the *LSK*1 enzyme in *Lotus japonicus*.

Key words: Lotus japonicus, CRISPR/Cas9 technology, Synthetic Biology, LSK1, nodulation, Golden Gate Assembly, Hairy root transformation

1. Introduction

Nitrogen is a nutrient of vital importance for plants, as it participates in plant growth, development and reproduction. Despite the fact that nitrogen is the most abundant element on earth, its deficiency is the most frequent occurrence that affects plant species worldwide. A tiny amount of organic nitrogen may exist in soluble organic compound forms in the soil, such as urea, that are not always available to plants. Atmospheric nitrogen is a major source of nitrogen into the soils. The nitrogen form contained in atmosphere is N_2, which must be converted in the soil in order to be useful and available to the plant roots.

To overcome this problem, several plants have developed and evolved a beneficial symbiotic relationship with soil bacteria that can convert N_2 into the plant-compatible form of nitrogen, ammonia (NH_3). These plants are called Legumes (*Leguminosae* or *Fabaceae* family) and their nitrogen fixing symbionts, rhizobia. For this reason, legumes hold a uniquely important position among crop plants that play a major role in soil nitrogen enrichment, and possess seeds rich in proteins, valuable in human and animal nutrition (Oldroyd et al. 2011).

Throughout legume-rhizobia symbiosis new organs are developed in the plant roots, called nodules. In general, nodules are the appropriate environment in which rhizobia can differentiate into bacteroids in order to fix atmospheric nitrogen and convert it into NH_3, a form that can be utilized by the plant metabolism. Not only plants, but also rhizobia gain from these plant-microbia interactions, as they receive carbon sources, useful for their life cycle.

The formation and maintenance of the newly developed organelles, called nodules, can be resource consuming; thus the plant symbiont has to regulate the total number of nodules to be formed post inoculation with the rhizobium. To accomplish this task, legumes have developed an endogenous system called Autoregulation of Nodulation (AON). In AON, plants possess a systemic function, as both the shoot and the root take part in the production of signals responsible for autoregulation of nodulation events. (Caetano-Anolles and Gresshoff 1991; Caetano-Anollés and Gresshoff 1991; Delves et al. 1986; Ferguson et al. 2010)

Very recently, a work conducted in the laboratory of Plants and Environmental Biotechnology, unveiled that nitrates play a major role in nodulation and subsequently in the activation of the AON mechanism. In the same work, a new *Lotus japonicus* Shaggy like kinase (*LSK*-1) was discovered and extensively studied. *LSK*-1 seems to play either a direct role in AON or an indirect one by regulating AON's components. Phenotypically, *LSK*-1 LORE insertion-mediated knocked out line, resulted a statistically significant increase in nodule numbers compared to the wild type plants (Garagounis et al. 2019).

Based on these findings, and aiming to optimize a gene knockout system, we developed a strategy, suitable to *Lotus japonicus*, and able to commit locus-specific genome editing using the CRISPR/Cas9 system to target *LSK*-1 gene.

1.1 CRIPR/Cas9 genome editing technology

In recent years, a wide range of genome editing technologies has emerged including TALENs (transcription activator-like effector nucleases), ZFNs (zinc finger nucleases) and CRISPR/Cas technology (Shim et al. 2017).

- TALENs and ZNFs are based on a strategy in which catalytic domains of endonucleases are combined with DNA-binding proteins in order to induce double-stranded breaks (DSBs) at certain genomic loci (Wood et al. 2011) (Figure 1).
- On the other hand, CRISPR/Cas is an endonuclease-small RNA coupling system in order to achieve targeted DSBs guided by Watson-Crickbase pair complementarity (Figure1).

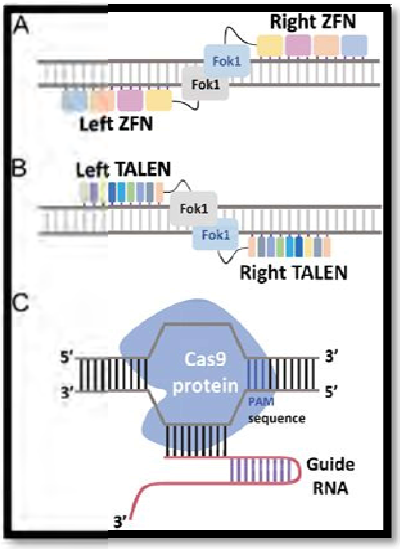

Figure1 A) ZNF, B) TALEN, C) CRISPR/Cas9 : the three most common nuclease-mediated genome editing technologies (Shim et al. 2017)

1.1.1 Genome editing utilizing nucleases

When nuclease-induced DSB occurs, the target region undergoes an endogenous pathway of DNA repair after damage. In almost all cells two repair pathways are active and further utilized by the cell to repair nuclease-mediated DSBs (Figure 2):

1. **NHEJ (Non-Homologous End Joining),** which is an **error-prone** pathway, repairs cleavage events in the absence of a repair template. The two DNA fragments are re-ligated through this process resulting in ***INDELS*** (random insertion/deletion mutations). Activation of this pathway is useful for:
    (a) ***gene knockouts***, because on *INDELS* within the gene's coding sequence(Perez et al. 2008) or
    (b) ***excision of a larger genomic region*** by targeting 2 neighbouring loci (Cong et al. 2013)

2. **HDR (Homology-Directed Repair)*,** a **high-fidelity pathway**, throughout which template-mediated repair occurs. The activation of this pathway can result in:
    (a) precise **modification** down to a single nucleotide in the presence of an exogenous DNA template,

(b) or even the **introduction** of a desired DNA sequence into the target genome

***Note**: In general HDR is active only throughout cell division with its efficiency to depend on the cell type, the target genomic locus and the repair template (Saleh-Gohari and Helleday 2004).

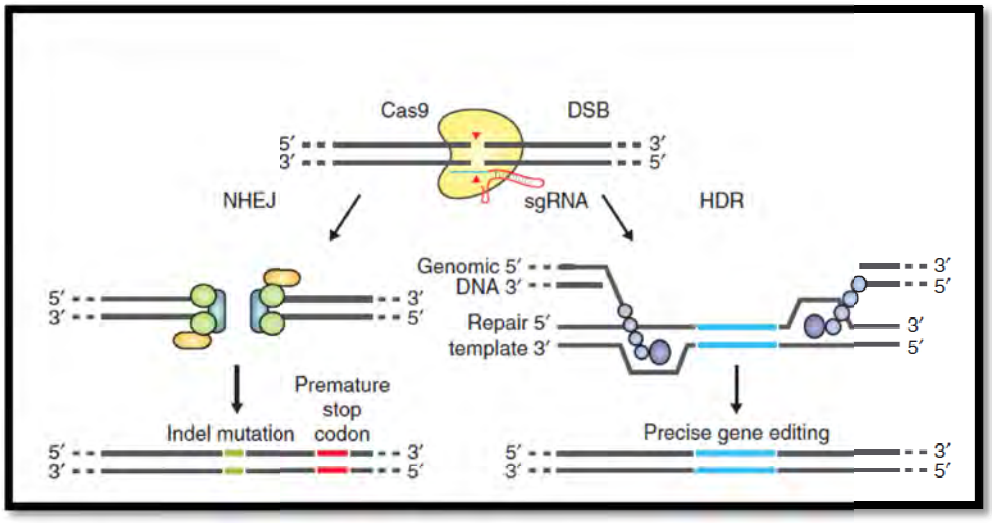

Figure2: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR) Endogenous DNA repair mechanisms(F Ann Ran et al. 2013)

1.1.2 CRISPR Cas system

Clustered regularly interspaced short palindromic repeats (CRISPR) coupled with CRISPR associated proteins (Cas), known as the **CRISPR/Cas system**, is an adoptive immune system encoded by many bacteria and archaea that helps them defend against foreign genetic elements, such as bacterial plasmids or viruses (Deveau, Garneau, and Moineau 2010; Horvath and Barrangou 2010).Three types of CRISPR/Cas systems have been identified and studied across archaea and bacteria (CRISPR/Cas I-III), but every single type is characterized by the following:

i. **a cluster of CRISPR associated genes**
ii. **non-coding RNAs**
iii. **and an array of palindromic sequence repeats**

These repeats frame 20 nucleotide variable sequences derived from foreign DNA, known as **protospacers** (Figure 3), this combination constitutes the CRISPR RNA **(crRNA)**(Makarova et al. 2011).

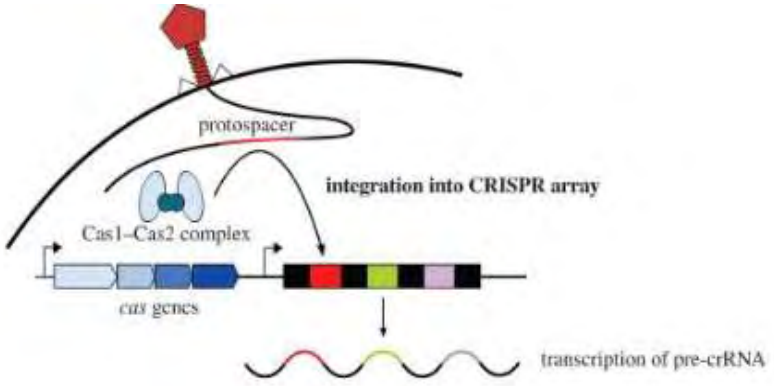

Figure3: Archea and bacterial adoptive immune system. The incorporation of protospacer (foreign DNA fragment) into host organism's genome is depicted. (Hille and Charpentier 2016)

The protospacer-encoded portion of the crRNA directs Cas nuclease to cleave complementary target-DNA sequences, if they are adjacent to short sequences known as **protospacer adjacent motifs (PAMs)** that are unique for every single type of CRISPR/Cas type (Brouns et al. 2008).

1.1.3 Type II CRISPR system

The most well studied and widely used CRISPR system is Type II, which is characterised as the "**minimal CRISPR-Cas system**" because of the presence of only three to four Cas genes at the Cas-genes-operon (Mir et al. 2018) (Figure 4).

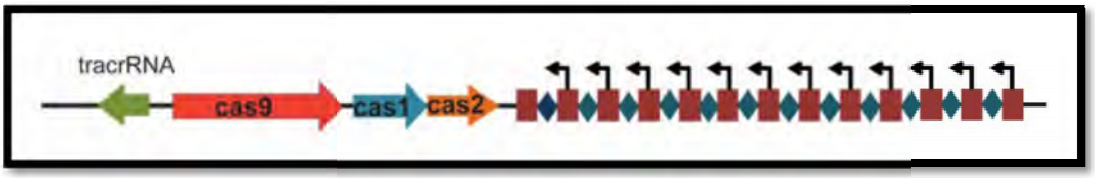

Figure4: Typical Type II CRISPR locus containing: tracrRNA, 3 Cas genes, CRISPR array. Diamonds: spacer sequences, Squares: repeats, Arrows: internal promoters (Mir et al. 2018)

Upon transcription of the CRISPR repeat arrays the nascent RNA is processed into **CRISPR RNA (crRNA)** that contains the spacer sequence and a part of the CRISPR repeat, which allows crRNA to hybridize with another RNA named **trans activating CRISPR RNA (tracrRNA)**(Garneau et al. 2010)(Figure 5)**.**

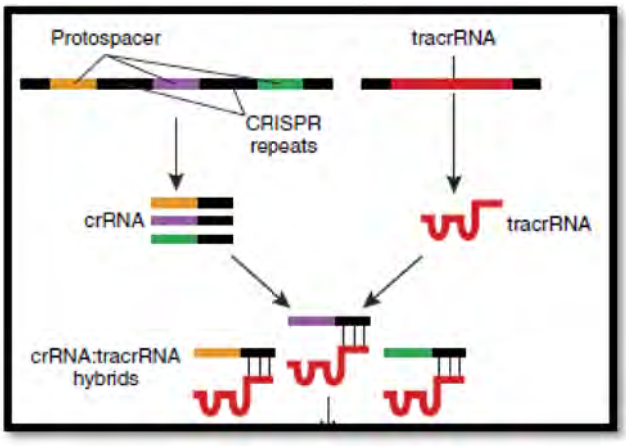

Figure5 CRISPR repeat arrays' transcription and production of crRNA and further hybridization with the tracrRNA (Sander and Joung 2014)

The crRNA:tracrRNA duplex creates a complex with the Cas9 nuclease, due to interaction of tracrRNA portion with Cas9. The spacer encoded portion of the crRNA recruits **Cas 9 to cleave the complementary foreign target-DNA sequence**, through its HNH and RuvC1-like nuclease domains, only if it is adjacent to a "signal sequence" named **protospacer adjacent motif (PAM**)(Jinek et al. 2012)*(Figure 6).

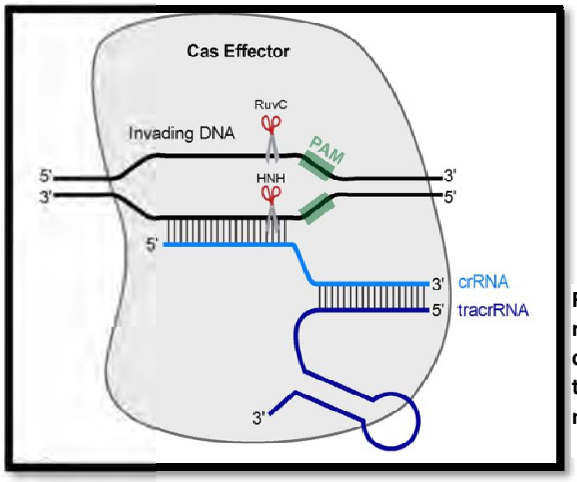

Figure6 : Depiction of a crRNA that recruits Cas9 nuclease to cleave the complementary DNA sequence through its HNH and RuvC1-like nuclease domains (Bier et al. 2018)

***NOTE**:In the CRISPR-Cas system derived from *Streptococcus pyogenes* (widely studied) the target DNA sequence is followed by a **5'-NGG PAM sequence**, but there are many Cas9 orthologs and each one of them requires an alternative PAM sequence(Cong et al. 2013; Sapranauskas et al. 2011; Zhang et al. 2013).

1.1.4 CRISPR/Cas9 system

The RNA-guided nuclease function of CRISPR/Cas9 system makes it a valuable research/diagnostic and therapeutic tool that belongs to the next generation of nuclease-mediated genome engineering. This system is now applied in order to introduce *INDELS* (via endogenous NHEJ) as well as to utilize endogenous HDR in the presence of either double stranded plasmid DNA or single-standed oligonucleotide donor template (ssODNs).

In the minimal, but most widely used form of CRISPR/Cas9 system, two modules must be incorporated into the cells or an organism in order to achieve precise genome editing:

i. **the Cas9 nuclease**
ii. **a guide RNA (gRNA)**

Cas9

Depending on the organism, a codon optimised Cas9 should be used in order to be functional. Furthermore Cas9 mutants have been widely used in order to further optimise the system and widen its applications. For example, Cas9 nickases, which have one of the two nuclease domains is disrupted, or dead Cas9 (dCas9), which has no nuclease activity at all (Hsu, Lander, and Zhang 2014)(Figure 7).

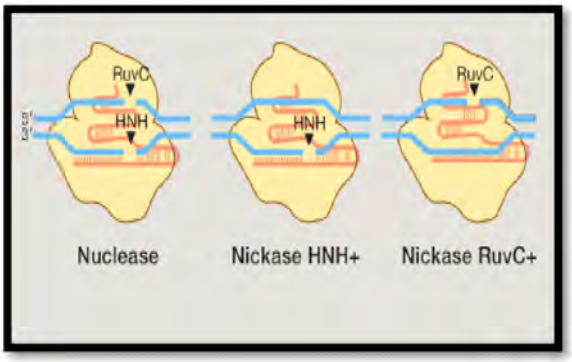

Figure7 Depiction of Cas9 nuclease and Cas9's nickases (Cas9 mutants) (Hsu et al. 2014)

gRNA

gRNA module utilized in genome editing, is a chimera of crRNA and an altered tracrRNA (gRNA scaffold), designed to avoid the dimerization of this two RNAs (Sander and Joung 2014) (Figures 8 and 9).

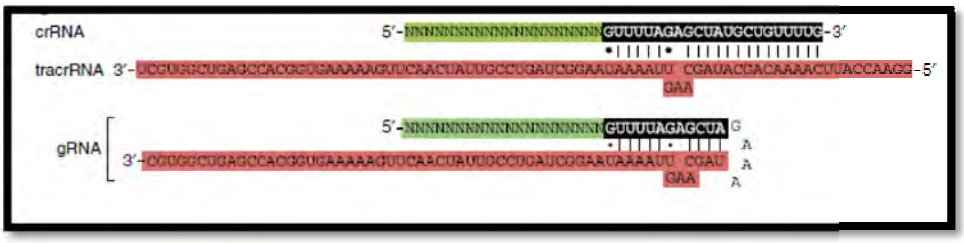

Figure8 Sequence comparison of two-components gRNA (crRNA hybridizing with tracrRNA) with the chimeric gRNA (crRNA and tracrRNA fusion) (Sander and Joung 2014)

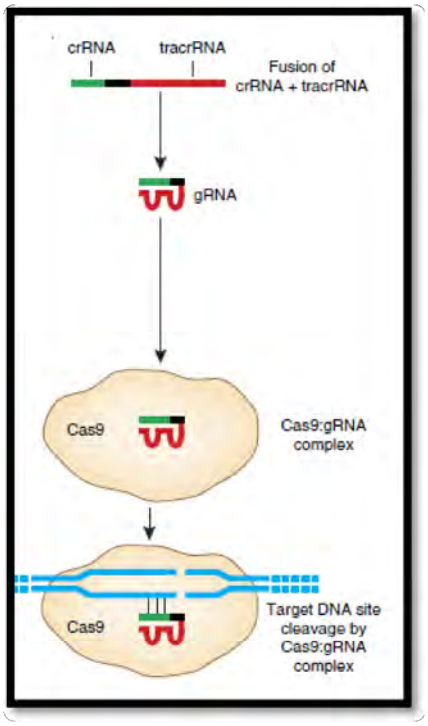

Figure9: guideRNA, a chimeric cr-tracrRNA utilized in modern CRISPR-mediated genome modifications (Sander and Joung 2014)

Within the last decade, a wide range of cell lines and organisms have been subjected to CRISPR/Cas9-mediated genome modifications. In the following **Table 1** there are some examples of the CRISPR/Cas9 system applications:

Table1 CRISPR/Cas9 applications among species

Cell type/organism	Cas9 form	Cell type	Citation
Human cells	dCas9	HEK293T,UMUC3,HeLa	(Chen et al. 2013)
Yeast	Cas9 nuclease	*S. cerevisiae*	(DiCarlo et al. 2013)
Mouse	Cas9 nickase	Embryos	(F. Ann Ran et al. 2013)
Zebrafish	Cas9 nuclease	Embryos	(Hwang et al. 2013)
Rice	Cas9 nuclease	Protoplasts,callus cells	(Shan et al. 2013)
Tobacco	Cas9 nuclease	Protoplasts, leaf tissue	(Li et al. 2013)

1.1.5 CRISPR/Cas9 system in plants

The very first attempts to employ CRISPR/Cas9 technology for plant genome editing, occur back to 2013, when researchers used transformation methods such as agro-infiltration and protoplast transfection, to create *INDEL* mutations in model plant species *A.thaliana*, *N.benthamiana* and *O.sativa* (Feng et al. 2013; Li et al. 2013; Nekrasov et al. 2013). Currently a variety of plant species have been modified by CRISPR/Cas9 system with a high success rate.

Cas9 gene and gRNA sequence can be introduced into the cells by conventional plant transformation methods, such as *Agrobacterium*-mediated transformation or biolistic method. The efficiency rate of CRISPR/Cas9-mediated genome modifications in plants depends on the following criteria:

i. **plant optimised Cas9:** usually by codon optimization based on plants' preferences and plant recognisable termination sequences (so far, both dicotyledon codon-optimized Cas9 and human codon-optimized Cas9 have been successfully used in legume plants with relatively similar efficiency) (Gasparis et al. 2018; Li, Zhang, and Sheen 2015).
ii. **Cas9 promoter:** Cas9 is usually driven by endogenous, PolymeraseII-dependent housekeeping genes' (*i.e*., ubiquitin gene) promoters, or a strong constitutive promoter like *Cauliflower mosaic virus* (CaMV) 35s promoter, or tissue specific etc. (C. Feng et al. 2018; Z. Feng et al. 2018).
iii. **Cas9 nuclear localization:** In all eukaryotic organisms should by fused with a Nuclear Localization Signal (NLS) for the efficient delivery of Cas9 into the nucleus (Lino et al. 2018).
iv. **gRNA promoter:** On the other hand gRNA's transcription is promoted by RNApol III dependent promoters, such as U6 or U3 snRNA's. More specifically, gRNAs driven by endogenous U6 or U3 promoter, seems to have higher expression levels than exogenous ones(Sun et al. 2015). If usage of endogenous U6 promoter is not possible, the promoters that can be utilized should only be from the same plant clade. In more detail, if one wants to study the effect of CRISPR/Cas9 system in a

dicotyledonous plant, U6 promoter should be derived by dicotyledonous species and for monocotyledonous plants by monocotelydonous respectively.

Currently, more and more scientific groups utilize the CRISPR/Cas9 system worldwide in order to modify plants genome. Except from this main goal, scientists also aim for the optimization of this tool; based on the species they are studying, in order to meliorate their findings within a more accurate framework. To give an example, nowadays crop plant models, like legumes, possess a prime position in the field of environmental and plant research. The necessity of their study stems from their significance both in environment and animal nutrition issues.

Within this framework Maekawa *et al* conducted a research comparing the transcriptional activities between the constitutively active CaMV35S promoter and the endogenous Ubiquitin promoter from *Lotus japoncus* in *Lotus japonicus* plants (Maekawa et al. 2008). Their findings indicate that there is an significant increase in expression levels utilizing the *L.j*. endogenous promoter, a work that is confirmed by experiments conducted in our Laboratory (Lab of Plant and Environmental Biotechnology) that show sub function of CaMV35S promoter both in *Lotus japonicus* and in related legumes like *Trigonella foenum-graecum*(unpublished data).

Concerning the previous statements, it is widely accepted that there is need of modern, specialized tools for making the study of such plants obtainable.

1.2 *Lotus japonicus*

Lotus japonicus is a model plant for the Legumes that used worldwide because of its characteristics. Legumes, or the ***Leguminosae* family**, consist of approximately 18,000 species, making it the third largest family of angiosperms. The most "popular" legumes are: soybean (*Glycine max*), pea (*Pisumsativum*) and beans (*Phaseolus vulgaris*) known for their importance as **crop plants**.

Figure10 *Lotus japonicus* flower (Plos blogs)

Lotus japonicus is grown in East Asia (Japan, Korea, China, India, Pakistan, Afganistan) and among all species two ecotypes,**Gifu' and 'Miyakojima MG20',** have been chosen for scientific studies.

Traits that make *Lotus japonicus* a legume model plant for classical and molecular genetic analysis are:

- Small genome size (~450 Mb)
- Diploid genome
- Six chromosome pairs
- Short seed-to-seed generation period
- Self-fertile flowers
- Large seed production levels
- Small seeds
- Large flowers (useful for manual crossing)
- Able to be transformed via *Agrobacterium tumefaciens* & *A. rhizogenes*
- Compatible with tissue culture and regeneration procedures
- Receptive to nodulation and mycorrhization

1.2.1 Nodulation

Most legumes have the capacity to interact with nitrogen-fixing soil bacteria, called ***Rhizobia*** **(belong in *Rhizobiacea*),** via beneficial endosymbiotic relationships. This profitable relationship is bilateral, as long as the plant has access to nitrogen fixed by *Rhizobia*, and the bacteria gains **carbon sources** derived from the plant. *Rhizobia* find accommodation in newly developed lateral root organs, called **nodules**, which are created right after inoculation of legume roots with the bacteria. Nodules, as lateral root organs, alter the root system architecture. For example, in *Lotus japonicus* after inoculation with ***Mesorhizobium loti***, the primary root growth is slower compared to the non-infected ones (Wopereis et al. 2000).

In order for root nodules to be formed, two processes are induced: a) **organogenic process** for the development of the new organs/tissues and b) **infection process** participating in bacterial colonization (Madsen et al. 2011). Nodulation is the phenomenon through which root nodules are developed utilizing epidermal, cortical and pericycle cells.

1.2.2 Nodule development and physiology

This interaction between the two symbionts is initiated on a subset of elongating root hair cells called **"susceptible zone".** In more detail the tips of root hair cells acquire a curved conformation to accommodate a small rhizobial colony. Furthermore, rhizobia colonize plants utilizing this conformation by creating an infection thread that invades into the cortex of the root (Held et al. 2010). As a result to a cascade of interactions, root cortical cells undergo dedifferentiation and many rounds of mitosis in order to form a nodule primordium (Geurts and Bisseling 2002) (Figure 12) . At the time an **Infection Thread (IT)** comes in contact with a nodule primordium, rhizobia are released into this newly-derived organelle domain (known as **nodule**), where they differentiate into **bacteroids** able to perform nitrogen fixation (Figure 11).

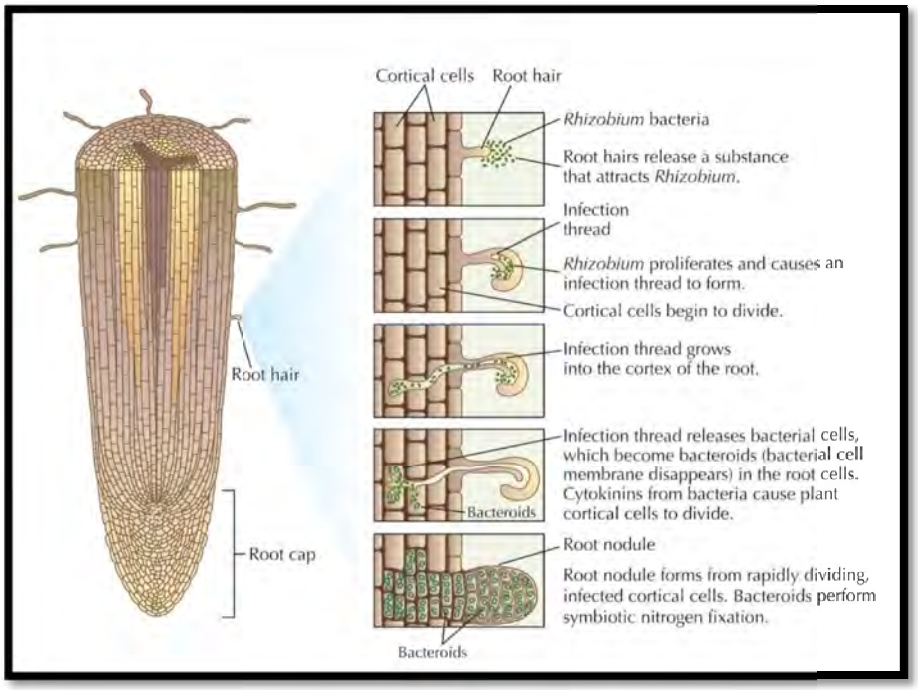

Figure11 Depiction of Root hairs infection with Rhizobium bacteria. Left: part of a root is shown and root hairs located at the infection susceptible zone. Right: root-rhizobium symbiosis stages. The following stages (from top to bottom)are depicted); a) root-rhizobium chemical recognition b) Infection thread development c) infection thread having effect on cortical cells d) Cortical cells proliferation and rhizobium differentiation into bacteroids e) mature nodule development (Nicholas H. Barton 2007)

Bacteroids are surrounded by the **symbiosome membrane (SM**) which is a natural barrier that separates the plant cells from the bacteroids, and concomitantly acts as a "nutrient conductor" that enables the exchange of solutes between the two symbionts, a procedure that is regulated and controlled by the host plant (Clarke et al. 2014).Finally, when the host plant has no longer great requirements of extra nitrogen, rhizobia are forced to stop nitrogen fixation and they no longer multiply. Subsequently, nodules stop to grow and they are getting non-functional via a natural aging process (Puppo et al. 2004).

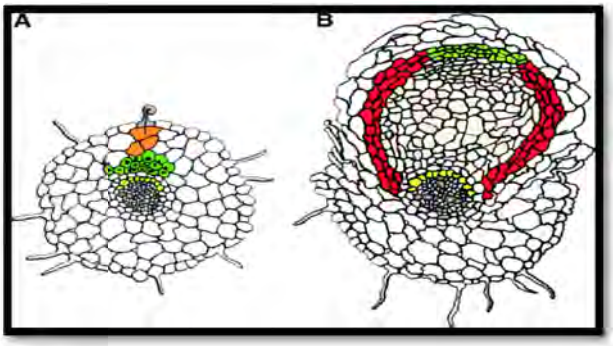

Figure12 A)Nodule premordium: Upon inoculation with the rhizobia, infection threads are induced by microcolonization in the curling conformation of root hair epidermal cells (blue). At the same time inner cell layers are activated. Pericycle cells undergo a limited number of cell mitosis (yellow). Cortex cells lose their identity and enter cell cycle which results the formation of a nodule primordium in the inner cortex (green). Outer cortical cells' cell cycle is arrested because they are going to form pre-infection threads (orange) B) Mature nodule: Nodule primordia are transformed into mature ones when rhizobia are released into inner cortex through infection threads. This new organelle consists of a central (pink) and a peripheral tissue (red). In addition to these two tissues a meristem is formed at the upper part of the nodule primordium (green). The central tissue (pink) is the one that going to host the rhizobia (Geurts and Bisseling 2002)

1.2.3 Nodulation's molecular base

The nodulation signal begins from the host plant, which secretes **chemical signals, flavanoids** that can be recognised by rhizobia. Once the signal is received by the bacteria, they produce lipochitooligo-saccharides, called **Nod (nodulation) factors (NFs)**, as a response to be received by the host-plant. In *Lotus japonicus*, Nod factors are recognised by specific receptors localized on root epidermal cell membranes (Radutoiu et al. 2003). Nod factor recognition by root cells is the initial and very important step of a series of molecular interactions in order for the nodulation to be established, such as regulation of early nodulation genes transcription, root hair cells curling and infection thread development (Gage 2004).

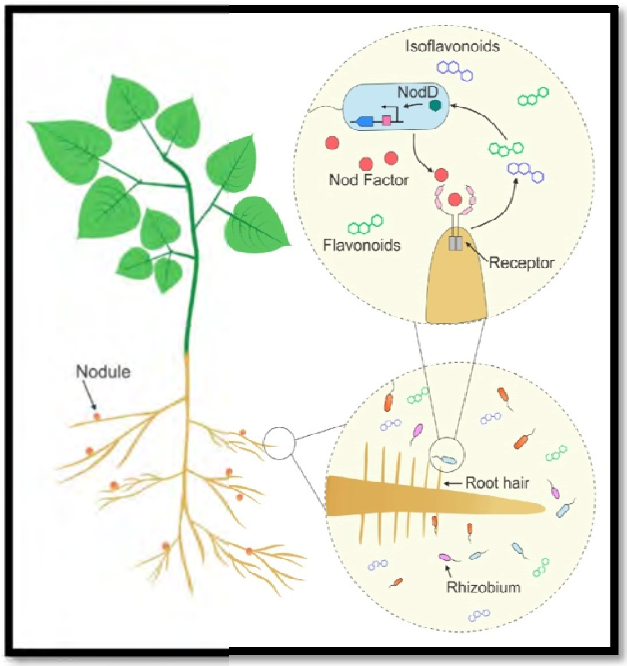

Figure13: Depiction of symbiosis initiation. Chemical recognition between the host plant and the Rhizobium at the roots of the plant (Joaquín Clúa et al. 2018)

In more detail, the molecular pathway that is responsible for the establishment of the nodules is the following:

1. Recognition of Nodulation Factors (NFs) by **Nod Factor receptors (NFRs**), which consist of a) an extracellular region containing two or three lysine motifs (LysM) domains, b) a transmembrane domain and c) a cytoplasmic kinase domain (Joaquín Clúa et al. 2018). In *Lotus japonicus* NFR1 and NFR5 heterodimerize to create a receptor which will only identify Nod factors produced by *Mesorhizobium loti* (Kouchi et al. 2010).
2. Additionally, another receptor, **SYMRK (symbiosis receptor-like kinase),** plays a major role in symbiosis initiation**.** This receptor consists of a) an extracellular region containing leucine-rich repeats domain (LRR), b) a transmembrane domain and c) a cytoplasmic kinase domain (Stracke et al. 2002). Even though SYMRK's ligand has not been identified yet; it is known that this receptor interacts with NFR5 in order to promote nodulation(Antolín-Llovera, Ried, and Parniske 2014).

3. Furthermore, the induction of a **calcium-mediated signalling pathway** is vital for nudulation. In *Lotus japonicus* five out of seven genes that consist the **"common symbiosis pathway" (CSP)** play a role in calcium induction into the root cells. These genes are: *SYMRK, CASTOR, POLLUX, NUP85 and NUP133* (Stracke et al. 2002).
4. The increased concentration of intracellular Calcium (Ca^{2+} spiking) leads to the activation of a Ca^{2+}/calmodulin-depented kinase (**CCaMK**) and the phosphorylation of the CYCLOPS protein by CCaMK.
5. The last step is the induction of transcription factor genes that play significant role in nodule establishment, such as: **GRAS,NSP1/2 and NIN** (Desbrosses and Stougaard 2011; Heckmann et al. 2006).

1.2.4 Auto Regulation of Nodulation (AON)

A molecular signalling pathway called Auto Regulation of Regulation (AON) controls systemic nodule development. This pathway is a negative feedback loop of molecular interactions, which allows the host plant to control nodule numbers in order to avoid excess nodule formation and further waste of sources.

Upon rhizobial infection, the production of two structurally similar peptides is induced in the root. These are encoded **by Rhizobia-Induced CLAVATA-Endosperm Surrounding Region (ESR)-related** (CLE) 1 and 2 in soybean. In *Lotus japonicus* these orthologues are called *LjCLE-RS1/2* (Ferguson et al. 2014; Okamoto et al. 2009; Reid, Ferguson, and Gresshoff 2011). In *Lotus japonicus* the genes are regulated by the transcription factor named **Nodule Inception (NIN),** but its role in the alterations of rhizobium-induced gene expression is not clear (Soyano et al. 2014). CLE-RS peptides are further modified via proline hydroxylation and subsequent glycosylation by an arabinosyltranferase called **NOD3/RDN1** (Corcilius et al. 2017; Hastwell et al. 2019; Okamoto et al. 2013). Several studies have indicated that CLE-RS modifications improve their stability by promoting the correct conformation, and increase the binding efficiency as a peptide ligand, but further experiments are required to reveal the precise role of these peptides (Hastwell et al. 2019; Shinohara and Matsubayashi 2015; Xu et al. 2015).

Downstream in the pathway CLE peptides are transferred **through the xylem to the shoot** and bind to a homo or heterodimeric receptor complex that is present at the parenchyma cells of the leaf vasculature (Nontachaiyapoom et al. 2007; Okamoto et al. 2009; Reid et al. 2011). This receptor is called **HAR1** in *Lotus japonicus*. In addition three other transmembrane proteins, CLAVATA2 (CLV2), CORYNE (CRN) and KLAVIER (KLV) are interacting with the CLE peptide-receptor. This interaction results in the auto-phosphorylation of the receptor by its kinase domain and the initiation of a cascade of interactions(Crook, Schnabel, and Frugoli 2016; Oka-Kira et al. 2005).

Even though the precise downstream signalling events are not known yet, experimental results confirm that this ligand-receptor interaction leads to the production of a **shoot-derived signal** that is subsequently **transferred through the phloem to the root.** This signal in turn acts as a suppressor of nodule formation by regulating a Kelch-repeat containing F-box protein, **Too Much Love (TML)**, which is located in the nucleus (Magori et

al. 2009; Takahara et al. 2013).Recently, it was confirmed that an RNA molecule is translocated from shoot to root in order to post-transcriptionally regulate TML in *Lotus japonicus*. This microRNA, **miR2111** acts as an activator of symbiosis in this negative feedback loop (Tsikou et al. 2018). Sasaki *et al.*, proposed another potential candidate for being the shoot-to-root signal of this pathway, the **Cytokinin hormones**, based on their results that showed alteration in the transcription levels of isopentenyl transferase in a HAR1-dependent manner *Lotus japonicus* plant (Sasaki et al. 2014).

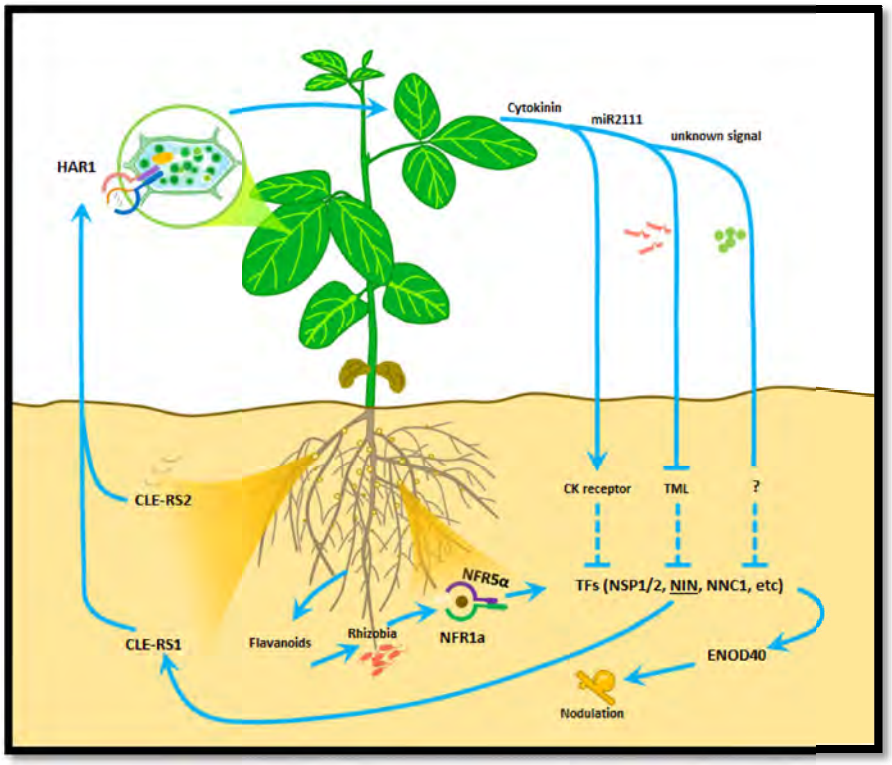

Figure14 Auto regulation of nodulation system depiction. In this figure there are mentioned both the root-to-shoot signals and the shoot-to-root ones (Ferguson et al. 2014)*modified

1.3 Glycogen Synthase Kinase 3 (GSK3)

Glycogen Synthase Kinase3 (GSK3), (also known as Shaggy Kinase (SK) in *Drosophila*) is a kinase that is widely conserved among eukaryotes (Saidi, Hearn, and Coates 2012). In the first place, it was identified as Ser/Thr kinase that plays a major role **in insulin's signaling pathway** by phosphorylating Glycogen Synthase in rabbit skeletal muscle cells (Cohen et al. 1982). Currently, in mammals, dozens of proteins have been identified as substrates for both GSK3 isoforms (GSK3α/β) which indicates that this enzyme could play a dominant role in a wide range of molecular pathways (Sutherland 2011). Detailed researches on GSK3s have shown that these enzymes are able to regulate important physiological and developmental processes, such as: metabolism, homeostasis, cell fate determination, embryo development,

neuronal differentiation, circadian rhythm etc (Doble 2003; Hur and Zhou 2010; Kim and Kimmel 2006).

1.3.1 GSK-3 variants

As mentioned previously in mammals there are **two GSK-3**, transcribed from separate genomic regions, that contain a much conserved kinase domain, but they differ at their amino- and carboxy- termini. **GSK-3α** contains a glycin-rich amino terminal extension, which is the reason why GSK3-α has a Molecular Weight of 51 kDa, in contrast with **GSK-3β** which has 47 kDa Molecular Weight. Moreover, there are two GSK-3β variants that differ in 13 amino acids (Saidi et al. 2012). In more detail, GSK-3β2 has a 13 amino acid insertion in the kinase domain but it is less abundant in mammals than the GSK3β1 variant (Schaffer, Wiedau-Pazos, and Geschwind 2003).

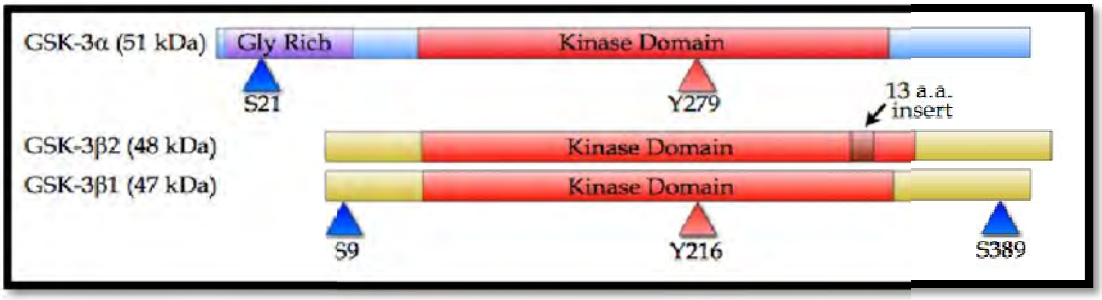

Figure15 GSK-3 genes products and isoforms of the enzyme. There are depicted the enzyme domain and the key residues that play a major role in GSK-3's regulation (Doble 2003)

1.3.2 GSK-3 regulation

As GKS-3s fold into their final conformation, in order to require full activity and, they undergo **autophosphorylation** acting as Tyrosin kinases, phosphorylating themselves on a key tyrosine residue located into the kinase domain(Lochhead et al. 2006).In GSK-3α this residue is **Tyrosine^{279}**, and in GSK-3β isoforms,**Tyrosine^{216} (Y216).**

On the other hand, phosphorylation events in C- or N- termini of the GSK-3 play an inhibitory role. More specific, when **Serine^{21}(S21)** of GSK-3α (or **Serine^{9}** of GSK-3β) in N-terminal, is phosphorylated by another kinase, it plays the role of an **intramolecular pseudosubstrate** that acts *in cis* as a competitive inhibitor, in order to "block" the activity of GSK-3(Cross et al. 1995).Another inhibitory phosphorylation event, that occurs near the C-terminus of GSK-3, has identified only for GSK-3βand not for GSK-3α. When Serine^{389} of mouse GSK-3β (or Threonine^{390} in human) is phosphorylated by another kinase, GSK-3 once again plays the role of a self-inhibitor acting as a **pseudosubstrate**(Thornton et al. 2008).

1.3.3 GSK-3 targets

GSK3s seems to have a preference in a specific type of substrates, which are molecules that have previously been phosphorylated from other kinases. In more detail,

GSK3 recognizes and **phosphorylates substrates that are pre-phosphorylated** at a standard position, which is four aminoacids C-terminal to the GSK-3 target site (Fiol et al. 1987).

Summarizing this information, we come to the conclusion that, the substrate sequence that can be recognised and subsequently phosphorylated by the GSK3 is the following one:

S/T-X-X-X-S/T

- First S/T : GKS-3 target, Serine or Threonine
- X: random residues
- Final S/T : pre-phosphorylation site

1.3.4 GSK-3's substrates

Frequently, it was indicated that substrates are phosphorylated more than once by the GSK-3. These substrates, contain in their sequence **tandem target motifs**, put in such a way that after the initial phoshporylation (pre-phosphorylation by another kinase), GSK-3 could phosphorylate consecutive target motifs located at N-terminal to the **pre-phosphorylation site,** with the ability to act as the priming kinase for each subsequent phosphorylation event (Figure 16).*In silico* analyses have unveiled hundreds of potential substrates for GSK-3 containing properly spaced tandem GSK-3 target-motifs, although, until 2011, less than 100 GSK-3 substrates have been reported in the literature as validated target molecules (Taelman et al. 2010).

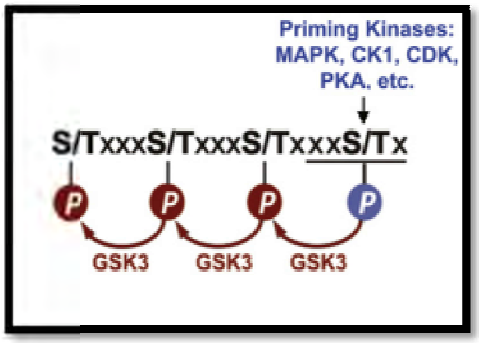

Figure16 Tandem GSK-3 target motifs. Multiple phosphorylation on GSK-3 after pre-phosphoryation by another kinase (Taelman et al. 2010)

1.3.5 GSK-3 in plants

Unlike in mammals, plant GSK3s are encoded by large multigene families whose members share a high rate of conserved sequences. So far, studies focused on angiosperms have shown that GSK-3 target phosphorylation motif is identical to those in GSK-3β, suggesting that plant GSK-3s are able to phosphorylate pre-phosphorylated substrates (Jonak and Hirt 2002).

In *Arabidopsis thaliana*, proteomic analysis has unveiled ten proteins that contain putative GKS-3 phosphorylation sites, and five of these were phosphorylated at the corresponding priming site (de la Fuente van Bentem et al. 2008). These ten GSK-3

homologues are named ***Arabidopsis thaliana*** **Shaggy Kinases (AtSKs),** shown n detail in the following **Table 2:**

Table2*Arabidopsis thaliana* homologous GSK-3 kinases (AtSKs) and their function

Arabidopsis GSK-3 clades	Arabidopsis GSK-3	Gene identifier	Function/Remark
I	AtSK11/ASKα	At5g26750	Flower development/brassinosteroid signalling
	AtSK12/ASKγ	At3g05840	Flower development/brassinosteroid signalling
	AtSK13/ASKε	At5g14640	Osmotic stress induced/brassinosteroid signalling
II	AtSK21/ASKh/BIN2/UCU1	At4g18710	Brassinosteroid signalling
	AtSK22/ASKi/BIL1/AtGSK1	At1g06390	Brassinosteroid signalling /salt stress
	AtSK23/ASKz/BIL2	At2g30980	Brassinosteroid signalling
III	AtSK31/ASKu	At4g00720	Brassinosteroid signalling/osmotic stress induced
	AtSK32/ASKb	At3g61160	Flower development
IV	AtSK41/ASKk/AtK-1	At1g09840	Unknown
	AtSK42/ASKd	At1g57870	Osmotic stress induced

In comparison with the mammalian GSK-3s, the plant SKs contain a highly **conserved sequence encoding the Kinase domain**, but **their N- and C- termini differ significantly**, a fact that interprets why different plant SKs are involved in **several biological processes**. For example, in *Arabidopsis thaliana*, C-terminal regions of *At*SKs are highly conserved because they play a major role in substrate recognition/interaction, but N-terminal sequences alter significantly affecting the subcellular localization(Kim et al. 2009; Youn and Kim 2015). Based on their amino acid sequences, plant SKs are clustered into four clades (I-IV) as shown in the following Figure 17 (Jonak and Hirt 2002).

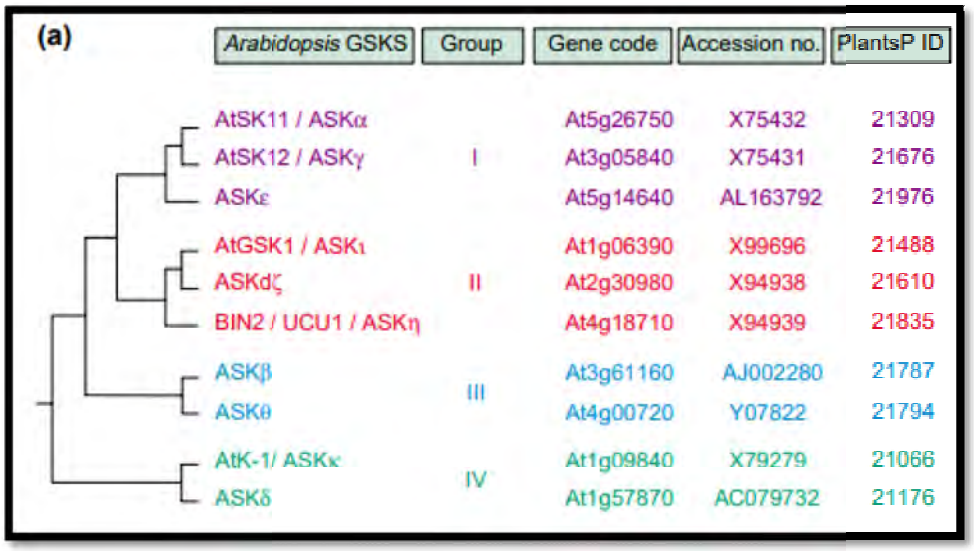

Figure17 Phylogenetic tree of *Arabidopsis thaliana*'s four SKs' clades(Jonak and Hirt 2002)

1.3.6 Role of "green" SKs

BR-Insensitive 2 (BIN2) is the first ever plant GKS-3-like kinase identified from genomic analysis (Li et al. 2001; Li and Nam 2002).Further biochemical analysis verified that BIN-2 is a plant Shaggy-Like Kinase that plays a negative **role in Brassinosteroids signalling**, and subsequently regulation of cell growth in *Arabidopsis thaliana*. Following this discovery, scientists were aimed to unveilethe substrates that are phosphorylated by the BIN2, and play a role in the BR signalling pathways. They accomplished to identify only two of them which are Brassinosteroid-responsive transcription factors [BRASSINAZOLE RESISTANT1 (BZR1), and bri1 EMS SUPPRESSOR1 (BES1)] in *Arabidopsis thaliana*(He et al. 2002; Yin et al. 2002; Zhao 2002).

A direct connection, at the molecular level, between Auxin and Brassinosteroids signalling pathways was indicated by a molecular interaction between AUXIN RESPONSE FACTOR 2 (ARF2) and the *At*SK21/BIN2 (Vert et al. 2008). Yeast two-hybrid screening showed a AtSK21/BIN-2 interacting protein to act as a transcriptional repressor in auxin pathway, that plays a regulatory role in *Arabidopsis thaliana* hypocotyls elongation. Summarizing, phosphorylation of this transcription factor (ARF2) by the *At*Sk21/BIN2 , inhibits the DNA binding and repressor activity of the ARF2 at the auxin response pathways (Vert et al. 2008).

Further studies conducted with plants, unveiled that SKs take place in several biological processes such as, regulation of root development and elongation, vascular system regulation, development of stomata and plant's reproductive organs and abiotic stress-responses (Youn and Kim 2015).

1.3.7 SKs in nodulation

Legume SKs have barely been studied under biotic or abiotic stresses, with no focus on the context of nodulation in a biotic-stress manner(Jonak 2000; Wrzaczek, Rozhon, and Jonak 2007). Until 2005, the only evidence of SKs implication in nodulation mentioned by(Kameshita 2005), when they cloned two *Lotus japonicus* SKs from nodule cDNA as a part of a wide screening in order to identify Ser/Thr kinases functioning in nodulation. Another fragment of a SK-like transcript performed a strong expression in *L.j.* nodules, compared to root tissue extracts (Colebatch et al. 2004).

1.3.8 *Lotus japonicus* SKs (*LSK*1-6)

Recent work unveiled six new protein coding regions that present sequence identity (70%) compared to the LNZ020/SK clone found by Kameshita et al., [sequence: AB113573.1 (GenBank, GI:62857011)] (Kameshita 2005),and a 76% homology with the *Arabidopsis thaliana*SKθ. These coding sequences, named *LSK*1-6 genes (*Lotus japonicus* Shaggy-Like Kinase), contain the conserved kinase domain sequence found in GSK-3β, an ATP binding site and the Tyr^{216} conserved residue that is also present in mammalian GSK-3β. All six *LSK*s differ in their N- and C- termini, with *LSK*1 and *LSK*6 having the longest N-terminal (approximately 40 amino acids) compared to the remaining *LSK*s (Garagounis et al. 2019).

Phylogenetic analysis of *LSK*s sequences clustered them into the four plant SKs clades as follows:

- *LSK*1,*LSK*6 : cladeIII

- *LSK*2, *LSK*5: cladeI
- *LSK*3, *LSK*4: cladeII

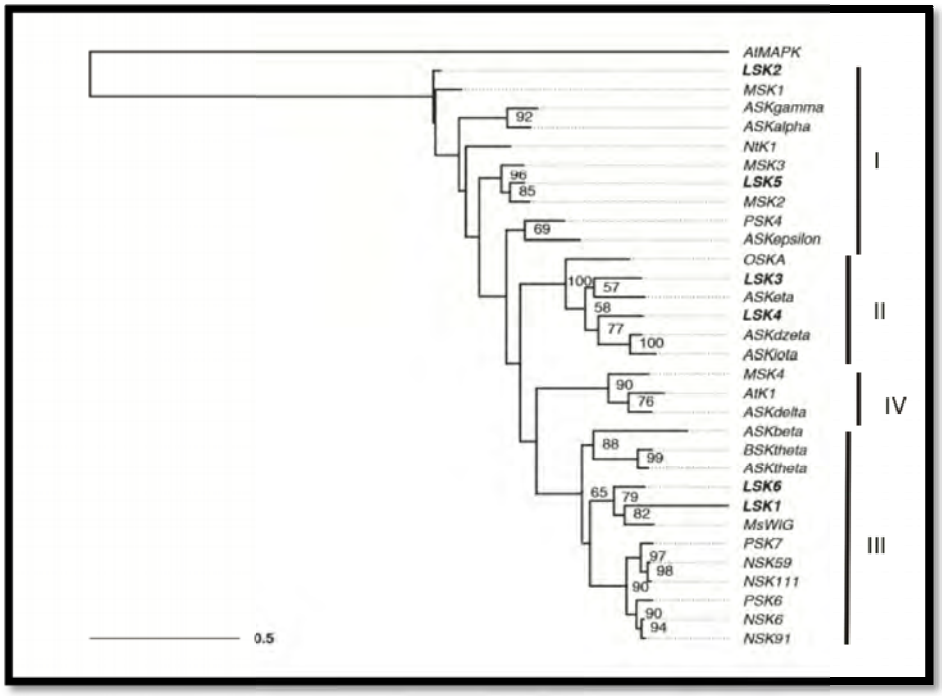

Figure18*LSK*1-6 phylogenetic clustering into plant four SKs clades (Garagounis et al. 2019)

1.3.9 The role of *LjLSK1* in nodulation

In the same research (Garagounis et al. 2019), the role of *LSK*1 was studied in detail. *LSK*1 seems to play a major role in the initial stages of nodulation, since it is present in roots, and in a smaller amount in leaves, at the first hour post inoculation with the rhizobacterium strain *Mesorhizobium loti* R7a. Gene silencing experiments of *LSK1* resulted in increased nodule numbers on roots (Figure 19).

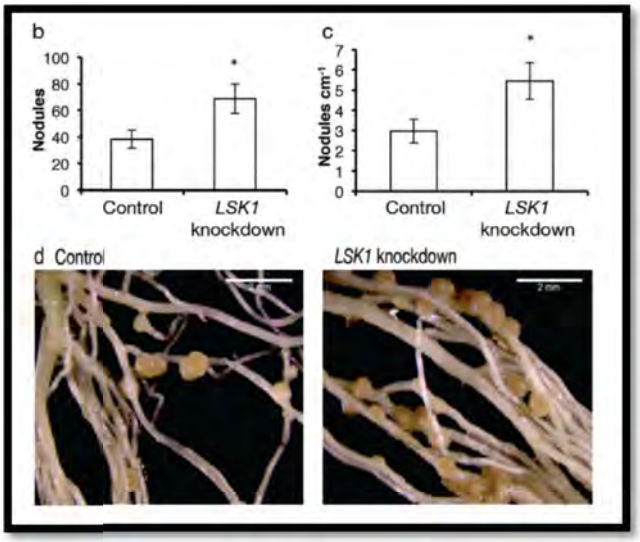

Figure19(b) Average ± SEM number of nodules and (c) Average ± SEM number of nodules normalized to root length of control (n = 5) and *LSK*1 - knockdown (n = 5) d) three week old regenerated hairy roots of control and *LSK*-1 knockdown plants (Garagounis et al. 2019)

On the other hand, ***LSK1* overexpression experiments** didn't present any statistical significant alternations in nodule numbers, indicating that *LSK1* is necessary but not sufficient for the inhibition of nodulation.

In order to identify the role of *LSK*1 in the Auto Regulation of Nodulation pathway in *Lotus japonicus*, further experiments were performed evaluating the gene expression levels of AON pathway key-molecules, in plants inoculated with the rhizobacterium.

In the absence, or at low Nitrate amounts, *LSK*1's transcription is induced 1hpi (1 hour post inoculation) by the rhizobacterium *M. Loti* R7a, and it is reported that it positively regulates the expression of NIN and NSP2 transcription factors, most probably via phosphorylation and subsequent activation/de-activation of intermediate factors.

On the other hand in the presence of high Nitrate amounts, *LSK*1's transcript levels are not altered but since it is a kinase it is likely to regulate other factors related to AON via phosphorylation. Subsequently activation of NIN transcription factor is inhibited, resulting the absence of CLE-RS peptide production. Under these conditions, CLE-RS2 production is induced by the activation of NRSYM1 (Nishida et al. 2018), an event that leads to an TML-independent nodulation inhibition.

In conclusion, it is not clear whether *LSK*1 is a protein-component of the AON pathway, but it is certain that it plays a major role in indirect regulation of key components that act in this pathway (Garagounis et al. 2019).

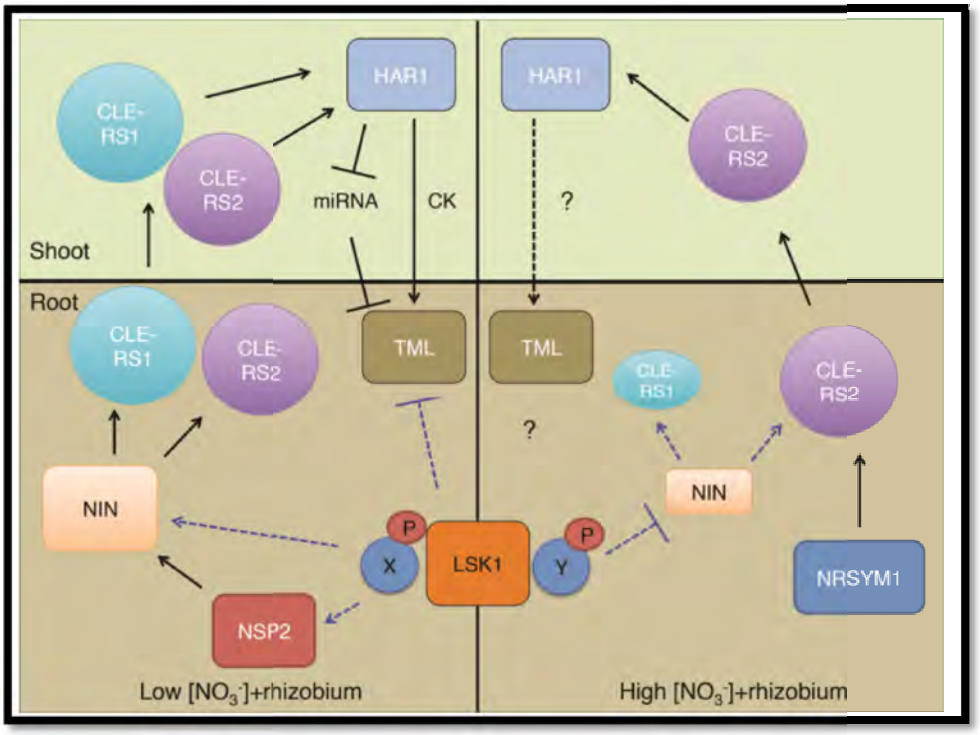

Figure20 Putative action of *LSK*1 under low or high NO_3^- concentration. Low concentration (left): NIN and NSP2 induction and further CLE-RS1/2 production.CLE-RS1/2 travel form root to shoot to interact with HAR1. Going on, shoot-derived miR2111 travels to the root to interact with TML. High concentration (right): *LSK*1-mediated activation of NRSYM1 leading to CLE-RS2 production. This pathway leads to TML-mediated nodulation inhibition. (Garagounis et al. 2019)

1.4 Thesis objectives

In this study, we focused on the optimization of CRISPR/Cas9 technology, in order to accurately trigger precise genome editing in the model plant *Lotus japonicus*. In more detail, we proceeded to a series of cloning steps in order to construct a **gRNA-acceptor binary vector**, which is able to express any gRNA under the control of the endogenous RNApol III dependent ***Lj*U6 promoter**. This vector, along with a binary vector containing an optimised Cas9 expression cassette, will be utilised in ***Agrobacterium*-mediated plant transformation.** Finally, we tested this newly constructed system in *Lotus japonicus* plants, by targeting the previously identified ***LSK*-1** gene. In order to accomplish this task, we focused on the transient expression of the **CRISPR/Cas9 system** in the roots, mediated by *Agrobacterium rhizogenes*-mediated hairy root transformation.

2. Materials and methods

2.1 Plasmid isolation (Miniprep)

1. Plasmid vectors were isolated from *E.coli* bacteria utilizing a **homemade alkaline lysis** protocol.

   For high copy plasmids:

   A 5ml of bacterial liquid culture was used. Cells were harvested in 1,5 ml tubes by centrifugation at 10,000g for 30sec. Then pellet was resuspended into 300µl of P1* buffer by vortexing and followed by the addition of 1 volume P2* and 1 volume P3* buffer. After 10 min incubation on ice, centrifugation followed for 10 min at 10,000 g and the supernatant was transferred into clean 1,5 ml tube. 0.66 volume isopropanol was added to the solution and spinned for 15min at 10,000 g. After discarding the supernatant, the pellet was washed with 500µl of 70% Ethanol by centrifugation at 10,000g for 10min. Last the supernatant was discarded and the pellet was dried for 5min at 65^{o}C. After that, DNA was resuspended in ~20µl ddH_2O and stored at -20^{o}C

   For low copy plasmids:

   10ml bacterial liquid cultures were used instead. Protocol's downstream steps where followed in the same manner except from the buffer volumes. In more detail 500µl of the buffers where used and 1ml of isopropanol.

   *You can find buffers recipes at supplementary material.

2. For plasmid **vectors required extra purification** (plasmids used for cloning/sequencing) NucleoSpin Plasmid- plasmid Miniprep kit of Macherey Nagel, was utilized by following manufacturer's instructions

2.2 DNA extraction protocol

For DNA extraction of *Lotus japonicus* leaf tissue the following protocol was utilized:

The tissue was grinded into 1.5ml tubes in presence of liquid Nitrogen. Then, 100µl of CTAB* solution (65^{o}C pre-warmed) was added with 1µl of mercaptoethanol, and incubated at 65^{o}C for 15min. After the addition of 150µl of chloroform isoamylic alcohol 1:25, the solution was spinned in centrifuge for 5min at 13,000 rpm. Following, upper phase was transferred into a clean 1.5ml tube and 0.7% volume isopropanol was added. After incubation of the solution for 10min in room temperature, centrifugation followed for 15min at 13,000 rpm. Then the supernatant was discarded and the pellet washed with 500µl of 70% Ethanol (10 min at 13,000rpm). At least the Ethanol was discarded and the DNA pellet dried at 65^{o}C for 5min. Then the pellet was resuspended in 50µl ddH_2O + RNAse and stored at -20^{o}C

*You can find buffers recipes at supplementary material.

2.3 *Escherichia coli* transformation

***E. coli* DH5α**

For each plasmid vector to be transformed, one 1.5ml tube of 80µl chemi-competent cells was used. After addition of ~100ng of the plasmid, the tube was put on ice for 30min. After that heat shock was followed for 55sec at 42^{o}C, and further incubation for 5min on ice. Then 1ml of LB* medium was added (pre-warmed 37^{o}C) and incubated on 37^{o}C for 45min. Last, cells were harvested and resuspended into 200 µl of fresh LB medium, and then 20µl and 180µl were plated in LB agar petri dishes in the presence of the appropriate antibiotic**. The plates were incubated overnight at 37^{o}C

Blue white screening

For transformation of cloning products utilizing a plasmid containing a LacA cassette, the whole transformation procedure was followed except from an alternation in the last step, where 50µl Xgal and 10µl IPTG were added.

2.4 *Lj*U6p cloning/domestication

2.4.1 *Lj*U6p amplification

For the amplification and domestication of the *Lotus japonicus* U6 promoter, the promoter was amplified in three fragments. As matrix for the PCR*, *Lotus japonicus* extracted genomic DNA was utilised. For the PCR reaction we used Q5 HF Polymerase 2x Master Mix (New England Biolabs). The PCR products were confirmed by gel electrophoresis in 1% agarose gel with the use of 2-log DNA ladder (New England Biolabs,) and further the products were purified with PCR clean-up Gel extraction - Macherey Nagel kit following the manufacturer's instructions.

*Primers and PCR conditions can be found at supplementary material.

2.4.2 Level-1 cloning

Two cloning reactions were performed at this step. First was the insertion of *Lj*U6p fragment1 into the pAGM1311 level-1 vector, and second the insertion of the *Lj*U6p fragment 2+3 fusion product into the pAGM1311 level-1 vector. All the components when cut with BsaI typeIIS restriction enzyme leave the following overhangs:

Component	Left (5' → 3')	Right (5' → 3')
***Lj*U6p fragment1**	ACAT (+strand)	ACAA (-strand)
***Lj*U6p fragment2**	ACAT (+strand)	CATC (-strand)
***Lj*U6p fragment3**	GATG (+strand)	ACAA (-strand)
pAGM1311	ATGT (-strand)	TTGT (+strand)

So, two one-pot reactions were performed, each one containing the following components: a)pAGM1311, b) *Lj*U6pfragment 1 (or 2+3), c) BsaI (NEB), d) Cutsmart buffer (NEB), e) T4 ligase (NEB) and f) T4 ligase buffer (NEB)*. Cloning products were transformed into *E. Coli* DH5α and plated in LB agar plates containing the appropriate selection*, Xgal and IPTG. Solid cultures were incubated over night at 37^{o}C.

*Cloning reaction conditions and antiobiotic concentrations can be found at supplementary material

Diagnostic digestions

pAGM1311:*Lj*U6p fragment1 and pAGM1311 vectors were cut into a BspHI (NEB) and NdeI (NEB) double digestion* in the presence of cutsmart buffer (NEB) and digestion products were visualised in 1% agarose gel with the help of the 2-log DNA ladder (NEB).

pAGM1311:*Lj*U6p fragment2+3 pAGM1311 vectors were cut into a DraIII (NEB) digestion* in the presence of cutsmart buffer (NEB) and digestion products were visualised in 1% agarose gel with the help of the 2-log DNA ladder (NEB).

*Detailed vector maps and restriction sites can be found at supplementary material

2.4.3 Level 0 cloning

In order to construct a level0 (promoter+5U module) vector containing the whole domesticated *Lotus japonicus* U6 promoter we preceded to another cloning reaction. When the components of this reaction cut with BbsI typeIIS restriction enzyme, the following overhangs are created:

Component	Left (5' → 3')	Right (5' → 3')
pAGM1311:*Lj*U6pfragment1	GGAG (+strand)	GCTT (-strand)
pAGM1311:*Lj*U6pfragment2+3	AAGC (+strand)	CATT (-strand)
pICH41295	CTCC (-strand)	AATG (+strand)

A single one-pot reaction was performed containing the following components : a)pICH41295, b) pAGM1311:*Lj*U6pfragment1, c) pAGM1311:*Lj*U6pfragment2+3, d) BbsI (NEB), e) NEB 2.1 buffer (NEB), f) T4 ligase (NEB) and g) T4 ligase buffer (NEB)*. Cloning products was transformed into E. Coli DH5α and plated in LB agar plates containing the appropriate selection*, Xgal and IPTG. Solid cultures were incubated over night at 37^{o}C.

*Cloning reaction conditions and antibiotic concentrations can be found at supplementary material

Diagnostic digestions

pICH41295:*Lj*U6p and pICH41295 vectors were cut into a BsaAI digestion reaction* in the presence of cutsmart buffer (NEB) and digestion products were visualised in 1% agarose gel with the help of the 2-log DNA ladder (NEB).

*Detailed vector maps and restriction sites can be found at supplementary material

2.5 gRNA scaffold cloning

2.5.1 gRNAscaffold amplification

As a matrix was utilized a gRNA vector containing the universal gRNAscaffold sequence, that was given to the laboratory by Dr Athanasios Dalakouras.The PCR* product was confirmed by gel electrophoresis in 1% agarose gel and was purified with PCR clean-up Gel extraction - Macherey Nagel kit following the manufacturer's instructions

*Primers and PCR conditions can be found at supplementary material.

2.5.2 Level 0 cloning

In order to construct a level0 vector containing the gRNAscaffold sequence we preceded to a one-pot cloning reaction. When the components of this reaction cut with BbsItypeIIS restriction enzyme, the following overhangs are created:

Component	Left (5' → 3')	Right (5' → 3')
gRNAscaffold	CTCA (+strand)	CTCG (-strand)
pAGM9121	TGAG (-strand)	CGAG (+strand)

The one-pot reaction was performed containing the following components : a)pAGM9121, b) gRNAscaffold, c) BbsI (NEB), d) NEB 2.1 buffer (NEB), e) T4 ligase (NEB) and f) T4 ligase buffer (NEB)*. Cloning product was transformed into E. Coli DH5α and plated in LB agar plates containing the appropriate selection*, Xgal and IPTG. Solid cultures were incubated over night at 37^{o}C.

*Cloning reaction conditions and antiobiotic concentrations can be found at supplementary material

Diagnostic digestions

pAGM9121:gRNAscaffold and pAGM9121 vectors were cut into a HinfI digestion reaction* in the presence of cutsmart buffer (NEB) and digestion products were visualised in 1% agarose gel with the help of the 2-log DNA ladder (NEB).

*Detailed vector maps and restriction sites can be found at supplementary material

2.6 gRNA_acceptor cloning

In order to construct a level 1 binary plasmid vector that contain the *Lj*U6promoter and followed by a universal gRNA cloning site fused with the gRNAscaffold we proceeded once again to a one-pot cloning reaction. . When the components of this reaction cut with BsaItypeIIS restriction enzyme, the following overhangs are created:

Component	Left (5' → 3')	Right (5' → 3')
pICH41295:*Lj*U6p	GGAG (+strand)	CATT (-strand)
pAGM9121:gRNAscaffold	AATG (+strand)	AGCG (-strand)
pICH47732	CGCT (-strand)	CTCC (+strand)

The one-pot reaction was performed containing the following components: a)pICH47732, b) pAGM9121:gRNAscaffold,c) pICH41295:*Lj*U6p, d) BsaI (NEB), e) cutsmart buffer (NEB), f) T4 ligase (NEB) and g) T4 ligase buffer (NEB)*. Cloning product was transformed into E. Coli DH5α and plated in LB agar plates containing the appropriate selection*, Xgal and IPTG. Solid cultures were incubated over night at 37^{o}C.

*Cloning reaction conditions and antiobiotic concentrations can be found at supplementary material

Diagnostic digestions

gRNA_acceptor and pICH47732 vectors were cut into a NdeI digestion reaction* in the presence of cutsmart buffer (NEB) and digestion products were visualised in 1% agarose gel with the help of the 2-log DNA ladder (NEB).

*Detailed vector maps and restriction sites can be found at supplementary material

2.7 gRNA:*LSK*-1target selection

For the selection of the appropriate gRNA for targeting *LSK*-1 gene we utilized the online CRISPR-P V2.0 platform which is found in the following website: crispr.hzau.edu.cn.

The genomic region that encodes for LSK1 is the following: **>chr0 chr0:32749920..32755086 (- strand)** and in was utilised as a query in order to obtain putative targets. The following table presents the selected gRNA characteristics, among them the on and off target scores.

sequence	region	strand	On score	Off target sites	greatest off score
ACCAAATCTACGTTGCACGGCGG	LSK1 exon11	-	0.8259	12	0.114

2.8 gRNA:*LSK*-1target cloning

2.8.1 oligo annealing

The two single strand 5' phosphorylated oligos (ssoligos) were hybridized following the downstream "oligo-annealing protocol" :equimolar amounts of each ssoligo was used in the reaction in the presence of T4ligase buffer (NEB) and the reaction was incubated in three cycles of 95^{o}C for 5min and room temperature for 20min.

2.8.2 Cloning reaction

The final gRNA:*LSK*-1target vector was constructed by performing another one-pot cloning reaction this time with the following components: a) 5' phosphorylated double strand oligo b) gRNA_acceptor c)SapI typeIIS restriction enzyme (NEB) d)cutsmart buffer (NEB) e)T4 ligase (NEB) and f) T4ligase buffer (NEB). Cloning product was transformed into *E. Coli* DH5α and plated in LB agar plates containing the appropriate selection*. Solid cultures were incubated over night at 37^{o}C.

2.8.3 Colony PCR

In order to identify the clones that contain the gRNA:LKS-1 target we performed a colony PCR. The components of this PCR were: a) single colony, b)dNTPs, c) forward and reverse primers*, d) Kapa taq 10X buffer (Kapa biosystems) and e) Kapa taq (Kapa biosystems). PCR products were visualized into 1.2% agarose gel with the help of the 2-log DNA ladder (NEB)

*Primers and PCR conditions can be found at supplementary material.

2.9 *Agrobacterium rhizogenes* transformation

All plasmid vectors utilized for hairy root transformation (pK7WG72::hCas9, gRNA_acceptor, gRNA:*LSK*-1target) were transformed into *Agrobacterium rhizogenes* strain LBA9402. The downstream freeze-thaw protocol was followed:

For each one of the plasmids a 5ml lquid culture of wild type *Agrobacterium rhizogenes* LBA9402 was incubated at 28°C and 160rpm overnight. Cells were harvested into 1.5ml tubes by centrifugation for 5min at 3,000 rpm. Then the cell pellet was washed three timed with ice-cold ddH_2O (centrifugation for 5min at 3,000 rpm) and dissolved into 15µl of 20mM $CaCl_2$ solution. Afterwards, the tubes were put into liquid nitrogen and followed thawing on ice for 40min. In continue, 1µg of each plasmid was added in separate Agro competent cells tubes, and put on ice for 5min. The next step was a freezing step in liquid nitrogen for 5min and after that incubation at 28°C for 3-4h. Last, cells were harvested and dissolved into 200µl of fresh LB medium. 2µl and 100µl were plated respectively in LB agar petro dishes in the presence of the appropriate antibiotic**. Incubation conditions for the cultures were, 28°C for 2days

*LB medium recipe can be found at supplementary material
** Antibiotics and antibiotic concentrations can be found at supplementary material

2.10 *Lotus japonicus* hairy root transformation

2.10.1 *L. japonicus* seed scarification/sterilization

600 seeds of *Lotus japonicus* ecotype Gifu were utilized for this experiment. The seeds were put into glass vials containing sulfuric acid (H_2SO_4) and incubated for 10min at room temperature by gently shaking. Then the acid was discarded and the seeds were washed 5 times with ice cold H_2O. Next, the seeds were incubated into 20%bleach-0.1% tween20 solution, for 18min at room temperature and further washed 8 times with ddH_2O. The vials containing the seeds and ddH_2O, were covered with aluminium foil and incubated at 4°C overnight

2.10.2 *L. japonicus* seeds germination

The next day seeds were transferred into 0.8% water agar petri dishes and then covered with aluminium foil and put at 26°C for 3 days. After three days, petri dishes were incubated into a 16h light/ 8h dark photoperiod at 26°C for three days

2.10.3 Hairy root transformation

After three days *Lj* seedlings were ready to be transformed by the *Agrobacterium rhizogenes*. In the following Table presented the amount of seedling utilized for each transformation event.

Purpose	vector transformed in A.r. strain LBA9402	*Lj* plants
CRISPR/Cas9 expression	pK7WGF2::hCas9/ gRNA:*LSK*-1target	120
Control	pK7WGF2::hCas9	60
Control	gRNA:*LSK*-1target	60
Control	gRNA_acceptor	60

For the transformation procedure we needed:

a) 12 solid cultures of *A.rhizogenes* LBA9402 transformed with pK7WGF2::hCas9, b) 12 solid cultures of *A.rhizogenes* LBA9402 transformed with gRNA:*LSK*-1target, c) 4 solid cultures of *A. Rhizogenes* transformed with gRNA_acceptor, d) 40 square petri dishes with ½ MS-agar medium, e) sterile ddH_2O and f) sterile forceps and blades and filter paper.

Each solid culture was used in order to transform 15 *Lj* seedlings except from the Cas9/gRNA cotransformation in which we used 1 solid culture from each Cas9 and gRNA:*LSK*-1target transformed *A.rhizogenes*.

The downstream **procedure** was followed:

2-3 ml of sterile ddH_2O were added at solid culture plate in order to suspend the *Agrobacterium*c ells. Alongside the *Lj* seedlings were transferred onto wet sterile filter paper and their primary root was excised by cutting right under the hypocotyl with a sterile blade. Next the wounded sites were inoculated with the *Agrobacterium* suspension and they were transferred into ½ MS-agar square petri dishes. The dishes were covered with aluminium foil and transferred at 26^{o}C for 4 days. After four days the dishes were uncovered and put at 16h light/ 8h dark photoperiod at 26^{o}C. After 2 days the seedlings were washed with sterile water containing 600μg/ml ceftazidime and transferred in fresh ½ MS-agar square petri dishes with 600μg/ml ceftazidime. 4 days later the plants were again transferred into fresh ½ MS-agar square petri dishes this time containing 800μg/ml ampicillin. This procedure was followed 2 more timed when finally the plants (24dpi) were potted into pots containing sand- vermiculite 2:1

The pots were kept at 16h light/ 8h dark photoperiod at 26^{o}C and the plants were watered every two days with dH_2O or Hoagland medium respectively.

2.11 *Lotus japonicus* plants sampling

39 days post hairy root transformation we sampled plant roots and shoots following the downstream procedure:

Roots and shoots of every single plant were measured in order to conduct a statistical analysis of their lengths. Plants were transferred into beakers filled with water in order to wash the vermiculite out of the roots. Next, the roots were separated from shoots and placed into separate 1.5ml tubes by freezing them immediately with liquid Nitrogen. Finally the tubes were stored at -80^{o}C

3. Results

gRNA_acceptor plasmid vector construction

A series of primers designing, cloning procedures, diagnostic digestions and sequencing events, were performed in order to combine bioparts in one single expression cassette, to construct a guide RNA acceptor site driven by the *Lotus japonicus* U6 promoter and followed by the guide RNA scaffold. In the next paragraphs you will find a detailed description of the steps followed to accomplish this goal.

3.1 *Lotus japonicus* U6 promoter domestication

*Lj*U6 promoter domestication means the construction of *Lj*U6p free of *BbsI* and *BsaI* recognition sites. The initial steps to accomplish this aim was to amplify the endogenous *Lj*U6p, from plant leaves, utilizing three sets of primers that possess single nucleotide mismatches within the type IIS restriction enzymes recognition sites. The primers that possess the mismatches are depicted in Figure 12 aligned with the *Lj*U6p sequence.

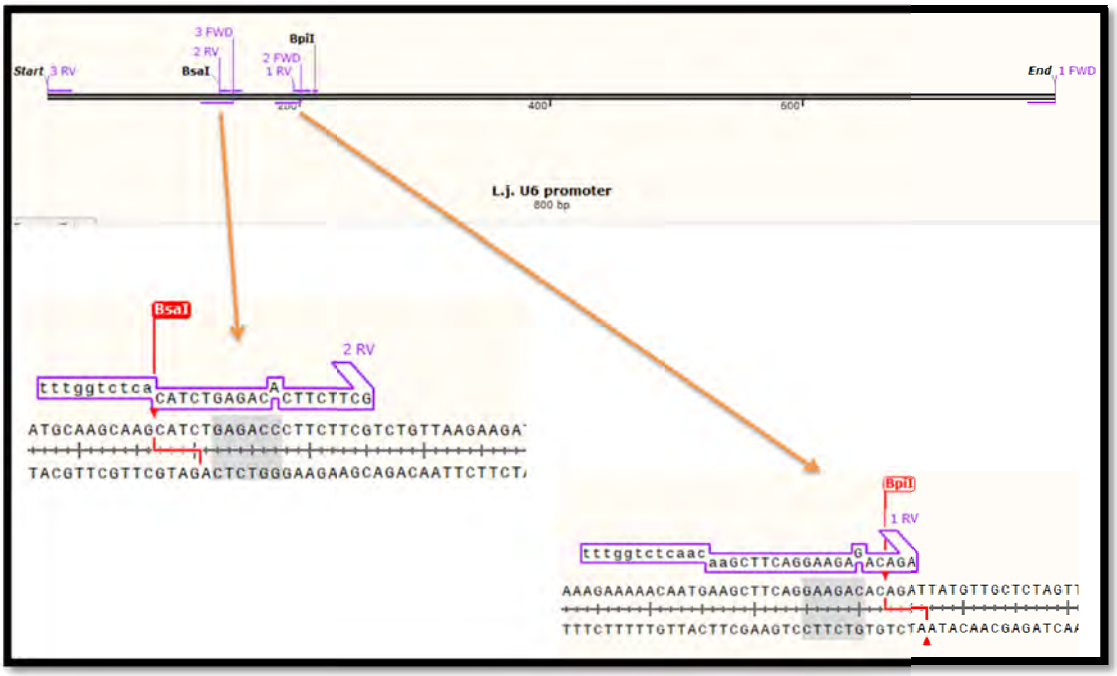

Figure21Three primer sets utilized for the *Lj*U6p amplification aligned with the *Lj*U6p sequence.

Except from the single nucleotide **mismatches**, these primers were designed to possess **flags** that are useful for the further cloning steps in order for us to achieve our final purpose, the gRNA acceptor cassette construction. The three primer sets designed for the *Lotus japonicus* U6 promoter amplification/domestication are the ones presented in Figure 22 and possess the following characteristics:

- ***Lj*U6p 1FWD**: a) *BsaI* recognition site for the cloning of fragment 1 into level-1 vector, b) level-1 fusion site, which is a four nucleotide overhang compatible with level-1 vector c) level 0 fusion, which will be a four nucleotide overhang compatible with level 0 and d) the initial nucleotides of *Lj*U6 promoter fragment1
- ***Lj*U6p 1RV**: a) *BsaI* recognition site for the cloning of fragment 1 into level-1 b) level-1 fusion site and c) the final nucleotides of *Lj*U6 promoter fragment1
- ***Lj*U6p 2FWD**:a) *Bsa*I recognition site for cloning of fragment2+3 into level-1 vector b) level-1 fusion site and c) the initial nucleotides of *Lj*U6 promoter fragment2
- ***Lj*U6p 2RV**: a) *BsaI* recognition site for fusion of fragment2 with fragment3 b) the final nucleotides of *Lj*U6 promoter fragment2
- ***Lj*U6p 3FWD:** a) *BsaI* recognition site for fusion of fragment 3 with fragment 2 and b) the initial nucleotides of *Lj*U6 promoter fragment 3
- ***Lj*U6p 3RV:** a) *BsaI* recognition site for cloning of fragment 2+3 into level -1 b) level -1 fusion site c) level 0 fusion site d) *SapI* recognition site, which is the half part of the gRNA acceptor site (it will be extensively described in later paragraphs) and e) the final nucleotides of the *Lj*U6 promoter fragment3

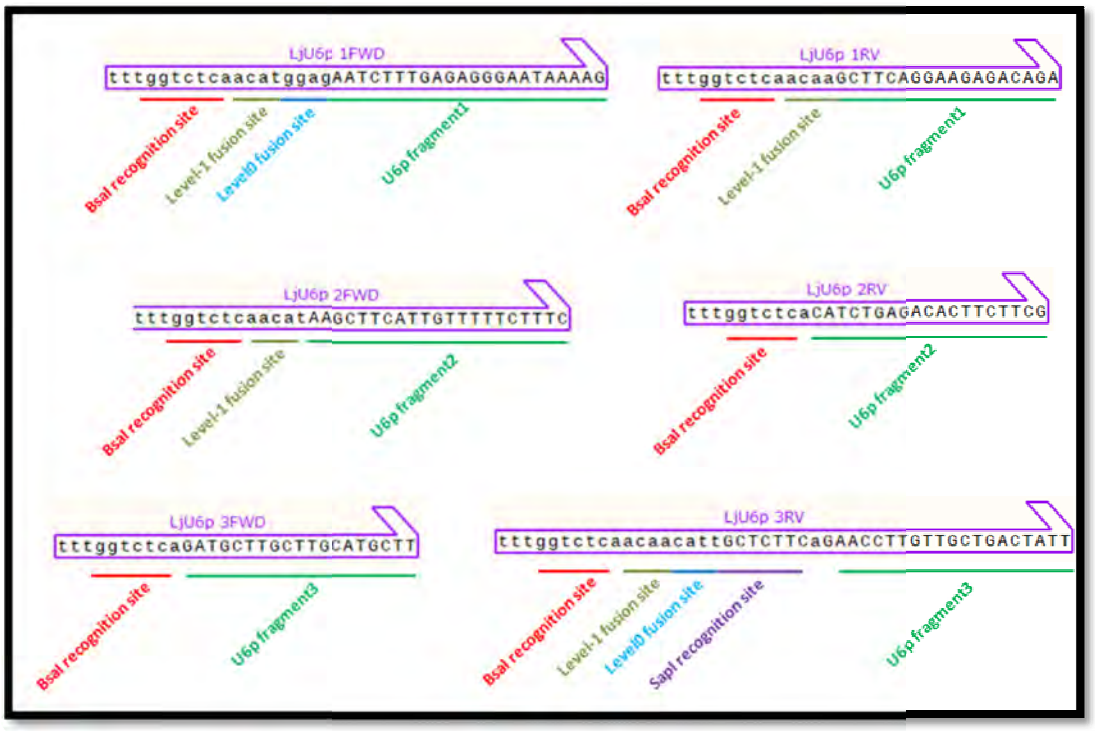

Figure 22 Determination of sequence features possessed by the three primer sets utilized for *Lj*U6promoter domestication

In Figure 23, the gel electrophoresis depicting the **amplification/domestication** of U6 promoter fragments, utilizing the previous sets of primers, and in the presence of a high

fidelity DNA polymerase, is shown. Lanes 1, 2 and 3 represents *Lj*U6p fragment 1, 2, and 3 respectively having the expected molecular weights (Figure 23).

Figure23*Lotus japonicus* U6 promoter fragment amplicons

Sequencing results have shown that indeed the *Lj*U6p fragments were amplified possessing the mutation within the recognition sites of *BbsI* and *BsaI* restriction enzymes. The following two sequence traces, highlighting in grey the point mutation within the recognition sites, support the fact that both the recognition sites have been eliminated, (Figure 24 and 25).

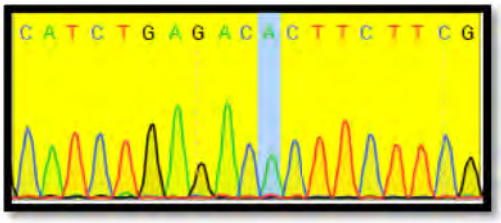

Figure 24 BsaI recognition site domestication (sequencing results proving the point mutation: highlighted nucleotide)

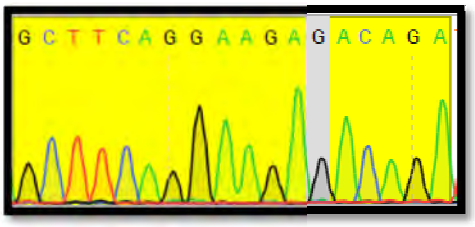

Figure 25 BbsI recognition site domestication (sequencing results proving the point mutation: highlighted nucleotide)

3.1.1 *Lj*U6 level-0 cloning

1. Cloning of *Lj*U6p fragment1 into pAGM1311 level-1 vector

Both the *Lj*U6p fragment 1 and pAGM1311 vector, when digested with *BsaI* restriction enzyme leave 4-nucleotide overhangs compatible with each other's **(Left fusion site: ACAT,**

Right fusion site: TTGT). In a one-pot digestion/ligation reaction (see details in Materials and Methods), pAGM1311 and *Lj*U6 fragment1 were digested with *BsaI* and ligated for the construction of pAGM1311:*Lj*U6pfragment1 level-1 vector depicted in Figure 26.

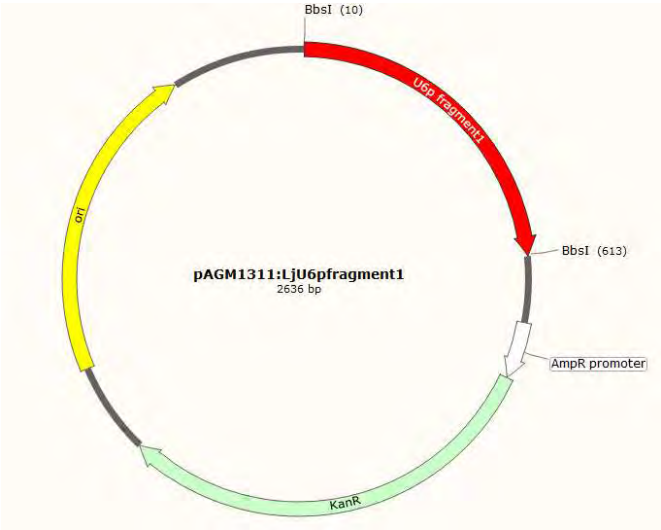

Figure26*Lotus japonicus* U6 promoter fragment 1 cloned into level-1 vector

After the digestion/ligation procedure, the newly constructed pAGM1311:*Lj*U6pfragment1 vectors were transformed into *E.coli* DH5a and cultivated into LB agar plates in the presence of Xgal/IPTG. At the following gel electrophoresis (Figure 27) is shown the diagnostic digestion of isolated plasmid vectors of 2 random white colonies (lanes 1-4), and the diagnostic digestion, with the same enzymes, of an empty pAGM1311 vector as control (lane 5). Only **clone 1** presented the expected band motif after digestion with BspHI and NdeI enzymes, verifying that clone 1 is the pAGM1311:*Lj*U6pfragment1 vector.

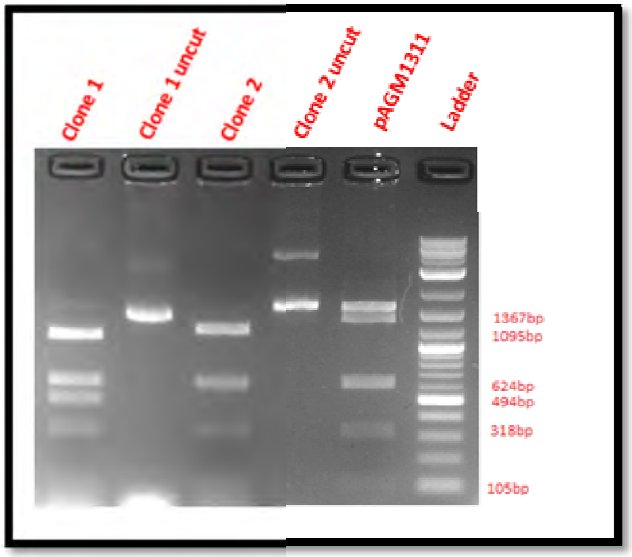

Figure 27 Diagnostic double digestion of 2 random clones containing the product of the *Lj*U6pfragment1-pAGM1311 cloning (lanes 1-4), and a control digestion of an empty pAGM1311 vector (lane 5). Restriction enzymes: BspHI and NdeI

2. Cloning of *Lj*U6p fragment2+3 into pAGM1311 level-1 vector

In this cloning procedure, the components were the *Lj*U6p fragment2, the *Lj*U6p fragment 3 and the pAGM1311 level-1 vector. When these nucleic acids were digested with *BsaI,* compatible overhangs were created for the fragment2 and fragment3 fusion **(GATG)** and the fragment2+3 insertion into the pAGM1311 vector **(Left fusion site: ACAT, Right fusion site: TTGT**). The constructed pAGM1311:*Lj*U6fragment2+3 vector is shown at the following Figure28.

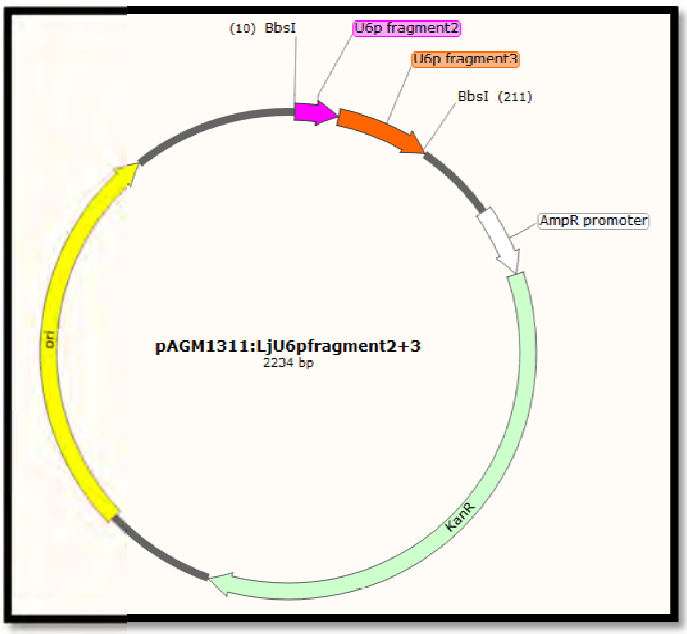

Figure 28 *Lotus japonicus* U6 promoter fragment2+3 cloned into level-1 vector

The following gel electrophoresis results of the diagnostic digestion (Figure 29), utilizing the same enzyme (DraIII), for both the clone (lane1) and the control (lane2), confirm the introduction of the *Lj*U6pfragment2+3 into the pAGM1311 level-1 vector because of the presence of the expected band motif in lane 1.

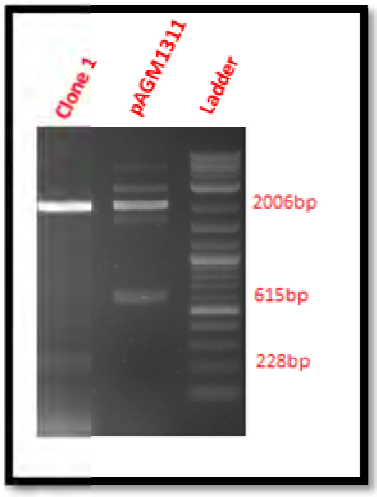

Figure 29 Diagnostic digastion of a random colony containing the *Lj*U6pfragment2+3-pAGM1311 cloning product (1) and of the empty pAGM1311 vector (control)

3. Cloning of domesticated*Lj*U6p into pICH41295 level0 vector

Aiming to this cloning, three components where utilized: a) pAGM1311:*Lj*U6pfragment1 b) pAGM1311:*Lj*U6fragment2+3 and c) pICH41295 level0 module. All of them where digested and ligated into the same one-pot reaction, with the use of *BbsI* typeIIS restriction enzyme (see Material and Methods) with which compatible overhangs were remained: (**fragment1 and fragment2+3 fusion site: GCTT)**, and for *Lj*u6p insertion into pICH41295: (**Left fusion site: GGAG, Right fusion site: AATG**). The cloning product was transformed into *E.coli* DH5a, in the presence of Xgal/IPTG. Because of the 4-nucleotide compatible overhangs left from the digestion, we accomplished to have the whole, free of *BbsI* and *BsaI* recognition sites, *Lotus japonicus* U6 promoter, into a pICH41295 level0 vector as depicted in the following Figure 30:

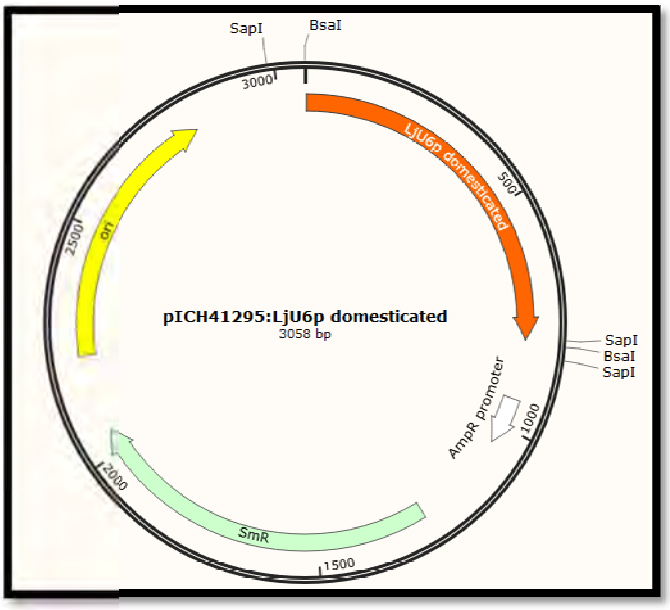

Figure30*Lj*U6promoter free of *BbsI/BsaI* recognition sites, into pICH41295 level0 module

We performed blue/white screening of the transformed *E.coli* DH5a cells, by selecting 12 white colonies that putatively contain the cloning product (pICH41295:*Lj*U6p). After liquid cultures and plasmid isolation, we compared the clones' molecular size compared with a control, which was the empty pICH41295 vector. The following results presented in Figure 31 show the 12 isolated clones (lane 1-12) and clones 5 and 7, highlighted with red arrows, contain most probably the correct pICH41295:*Lj*U6p vector because of the expected molecular size.

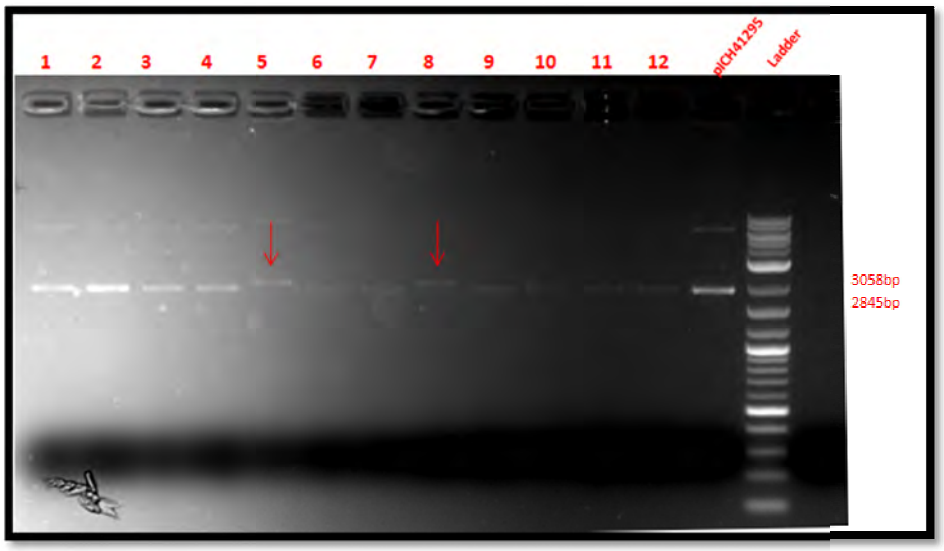

Figure 31 Gel electrophoresis of 12 clones containing the pICH41295:*Lj*U6p cloning product

In order to further confirm this, we performed diagnostic digestions. The following gel electrophoresis in Figure 32 presents the results of the recognition digestion of both a) clones 5 and 8 (lane 1-2) and b) the empty pICH41295 vector (lane 3). After digestion with the same enzyme (*BsaAI*, see Material and Methods), the resulting bands confirm the insertion of the whole *Lotus japonicus* U6 promoter into the pICH41295 level 0 module based on the desired band motif.

Figure32 Diagnostic digestion of clone 5 and 8 (pICH41295:*Lj*U6p) and empty pICH41295 vector

3.2 gRNAscaffold level-0 vector

gRNAscaffold do not contain any *Bsa*I or *Bbs*I recognition sites, thus there was no need to be domesticated by PCR-mediated mutagenesis. For the gRNAscaffold amplification the following primers where designed, which contain a flag consisting of:

- **gRNAscaffold 1FWD**: a) BbsI recognition site b) 4-nucleotide site for level0-gRNAscaffold fusion c) U6p-SapI3' fusion site) d) SapI recognition site and e) the initial nucleotides of the gRNA scaffold (c and d will extensively mentioned in next paragraphs)
- **gRNAscaffold 1RV**:a) BbsI recognition site b) 4-nucleotide site for level0-gRNAscaffold fusion and c) the final nucleotides of the gRNAscaffold

The sequence features of the designed primers are depicted in Figure 33

Figure 33 The primer set utilised for the gRNAscaffold amplification

In order to clone the gRNAscaffold into the level 0 vector, we had first to amplify it utilizing a High Fidelity polymerase. We used, as a matrix, a plasmid vector that was given to the laboratory by Dr Dalakouras, and it contained, among others, the gRNAscaffold sequence. At the following Figure 34, the purified gRNAscaffold amplicon of 161- nucleotide size is depicted.

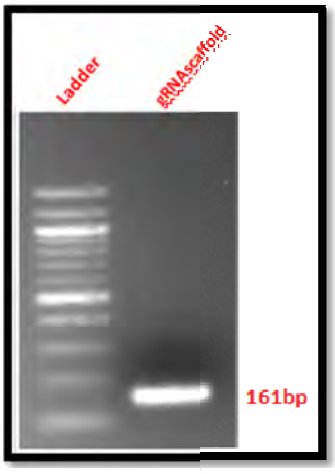

Figure 34 Gel electrophoresis of the gRNAscaffold amplicon

Then, we performed a one-pot digestion ligation reaction utilizing the *BbsI* typeIIS restriction enzyme. Within this reaction both gRNAscaffold amplicon and pAGM9121 were present, and when were digested with *BbsI*, 4-nucleotide compatible overhangs where created and ligated with each other in order for the pAGM9121:gRNAscaffold plasmid to be constructed, as shown in Figure 35.

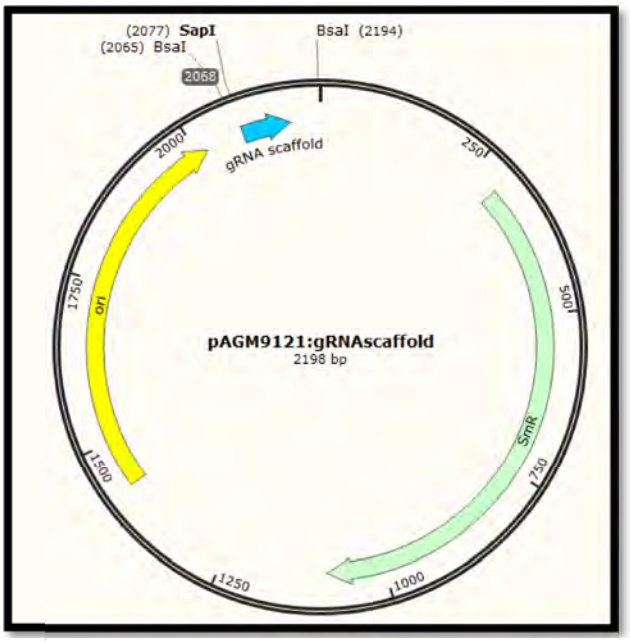

Figure 35 gRNAscaffold cloned into level 0 vector

The cloning reaction product was transformed into *E.coli* DH5a cells, and subsequent culture in LB agar plates in the presence of Xgal/IPTG. After blue-white screening, 4 random colonies were selected for liquid cultures and subsequent plasmid isolation. Figure 36 shows the diagnostic digestion of these 4 clones (lanes 2-9) and of the empty pAGM9121 vector (lane 10), utilizing the *HinfI* restriction enzyme. In gel electrophoresis, all the clones presented the expected band motif, and so introduction of the gRNAscaffold into the pAGM9121 level0 vector was confirmed to.

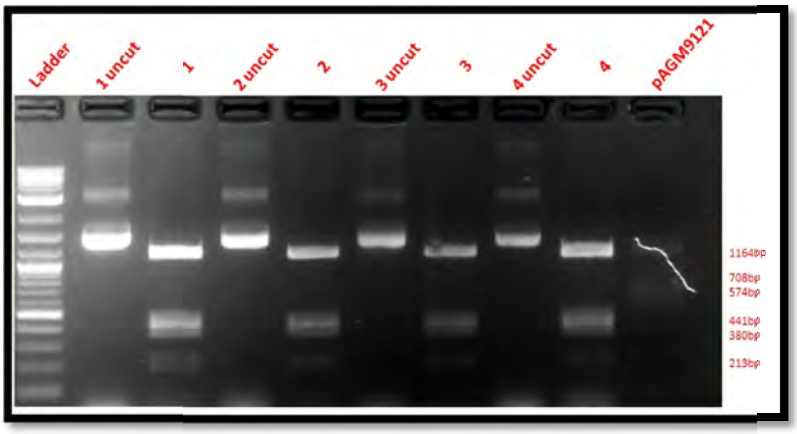

Figure36 Diagnostic digestion of 4 pAGM:gRNAscaffold clones and empty pAGM9121 vector, utilizing the HinfI restriction enzyme.

3.3 gRNA_acceptor vector level1

The last step of these cloning series was the construction of the final gRNA_acceptor vector, which is a plasmid vector that can host a 20nt guideRNA between the *Lj*U6promoter and the gRNAscaffold. To achieve this goal, we performed another one-pot digestion/ligation reaction, this time utilizing the *BsaI* restriction enzyme. The components needed for this reaction was the pICH41295:*Lj*U6p, the pAGM9121:gRNAscaffold and the pICH47732 level 2 module. In the following Figure 37, the pICH47732:*Lj*U6p-SapI-gRNAscaffold vector, named hereafter gRNA_acceptor, is shown. The compatible overhangs created by the *BsaI* digestion were: **for *Lj*U6p-SapI and SapI-gRNAscaffold fusion (AATG)** and for the insertion of gRNAacceptor into pICH47732**: Left fusion site AGCG, Right fusion site CTCC.**

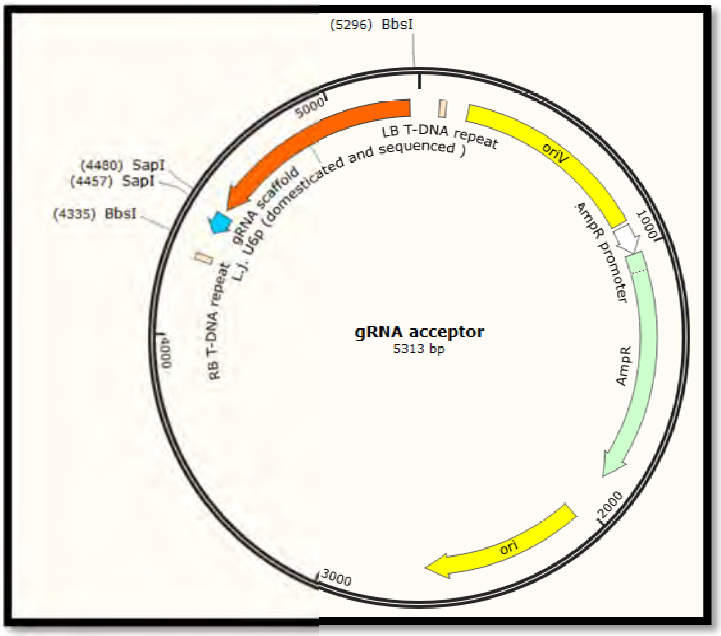

Figure 37 gRNA_acceptor binary vector containing the domesticated *Lotus japonicus* U6 promoter and a gRNA universal cloning site followed by the gRNAscaffold

Through blue/white screening we selected 8 white colonies for liquid cultures and subsequent plasmid isolation. Both these 8 clones (lane1-8) and the empty pICH47732 vector (lane 10) were utilized in a diagnostic digestion with *NdeI* enzyme, and the electrophoresis results shown in Figure 38 confirm, for lanes 1-7, that the whole gRNA_acceptor was cloned into the pICH41295 level1 module based on the presence of the expected band motif.

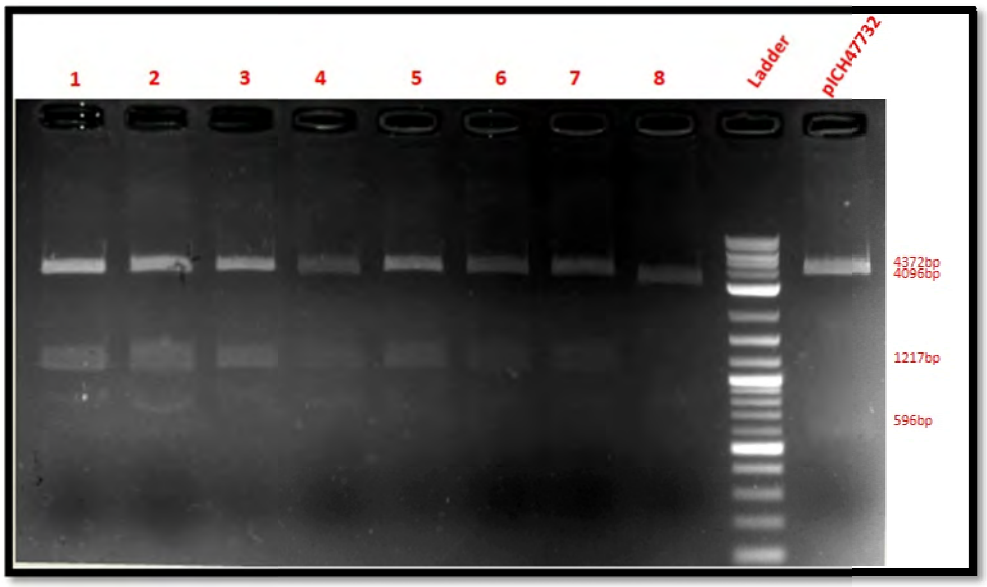

Figure 38 Diagnostic digestion of both 8 (1-8) gRNA_acceptorclones and an empty pICH41295 vector with NdeI restriction enzyme

The whole gRNA_acceptor cassette was sequenced (data not shown) confirming the correct arrangement and DNA sequence of the *Lj*U6p-SapI-gRNAscaffold module. In the following Figure 39 the "gRNA host" site between the *Lj*U6p and the gRNAscaffold, which is a cloning site for any gRNAoligo (panel A) as well its sequencing trace (panel B)are presented.

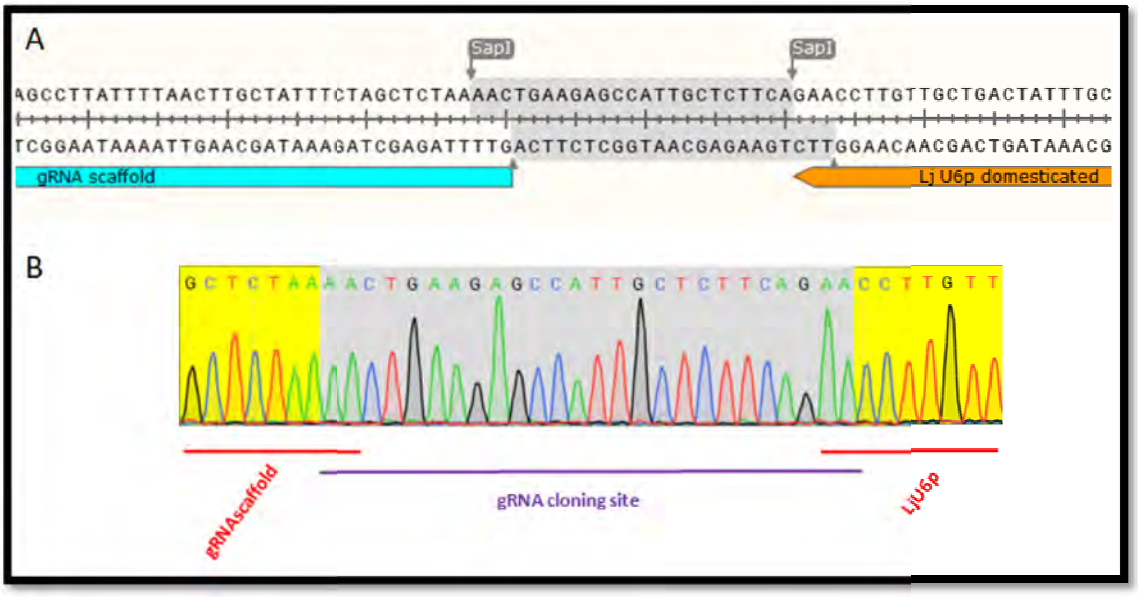

Figure 39 gRNA cloning site between *Lj*U6p and gRNAscaffoldA)cloning site depiction B) sequencing trace of the cloning site

3.4 gRNA for *LSK*-1 targeting

CRIPSR-P v2.0 online software helped us to select, among others, the suitable gRNAoligo for targeting *LSK*-1 gene. This gRNA is homologous to a 20nt region into *LSK*-1 exon 11.

The initial form of this gRNA olgonucleotide (oligo) was two single strand nucleic acids (5' phoshporylated), that after an standard annealing protocol, they hybridized with each other leaving 3-nt overhangs as shown at the following Figure 40 .

Figure 40 double strand gRNA oligo homologous to region at *LSK*-1 exon11

In order to construct the vector carrying this gRNA that targets *LSK*-1, we performed an one-pot cloning reaction in the presence of *SapI*typeIIS restriction enzyme. The two components utilized in this cloning procedure were: the 5' phosphorylated gRNA oligo and the gRNA_acceptor vector. After the digestion with the *SapI* restriction enzyme, the 3nt overhangs created at the gRNA_acceptor vector was ligated with the gRNA oligo's compatible ones **(Right fusion site: TTC, Right fusion site: GTT).** The following Figure41 depicts the gRNA:*LSK*-1target vector.

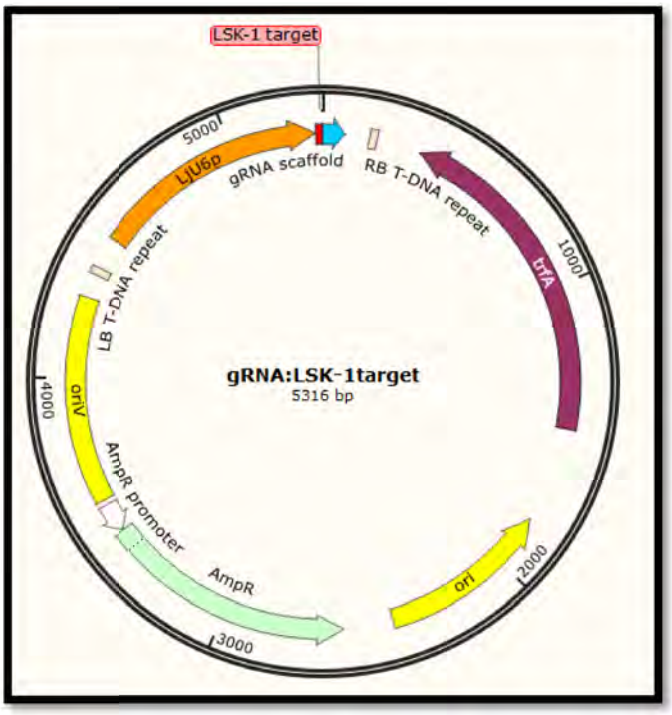

Figure 41 gRNA:*LSK*-1target, a binary vector for the expression of a gRNA aiming on *LSK*-1 exon11 targeting

The cloning product was transformed into *E.coli* DH5a cells and cultured in LB agar plates. This time there was no selection of blue/white screening, and for that reason a colony PCR was performed in order to confirm the *LSK*-1 target into the gRNA_acceptor, utilizing as forward primer the single strand oligo and as reverse, a primer that hybridizes with U6 promoter. In Figure 42, the Colony PCR results, indicating that all 8 clones (lanes 2-8) contain the desired oligo, are presented.

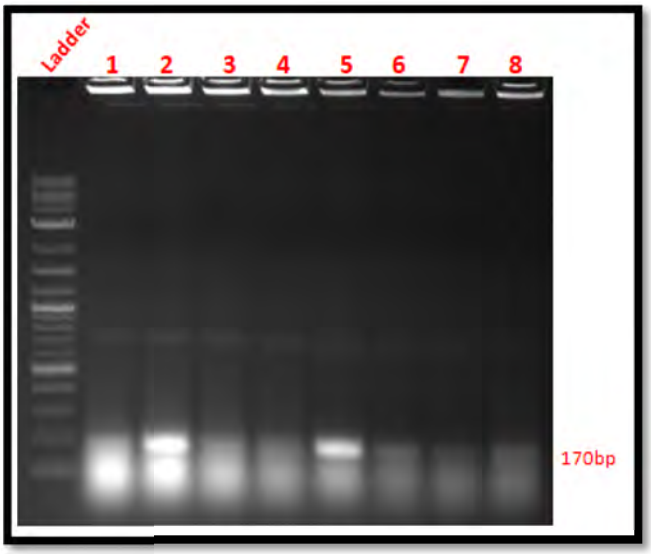

Figure 42 Colony PCR of 8 clones (1-8) of gRNA:*LSK*-1target vector

Then colonies 4 and 5 were inoculated in liquid cultures, and subsequently, plasmid isolation was performed. Both clone 4 and clone 5 were sequenced and in Figure 43 the sequencing trace of clone 5 that confirmed the introduction of the gRNA oligo between *Lj*U6p and gRNA scaffold, is presented.

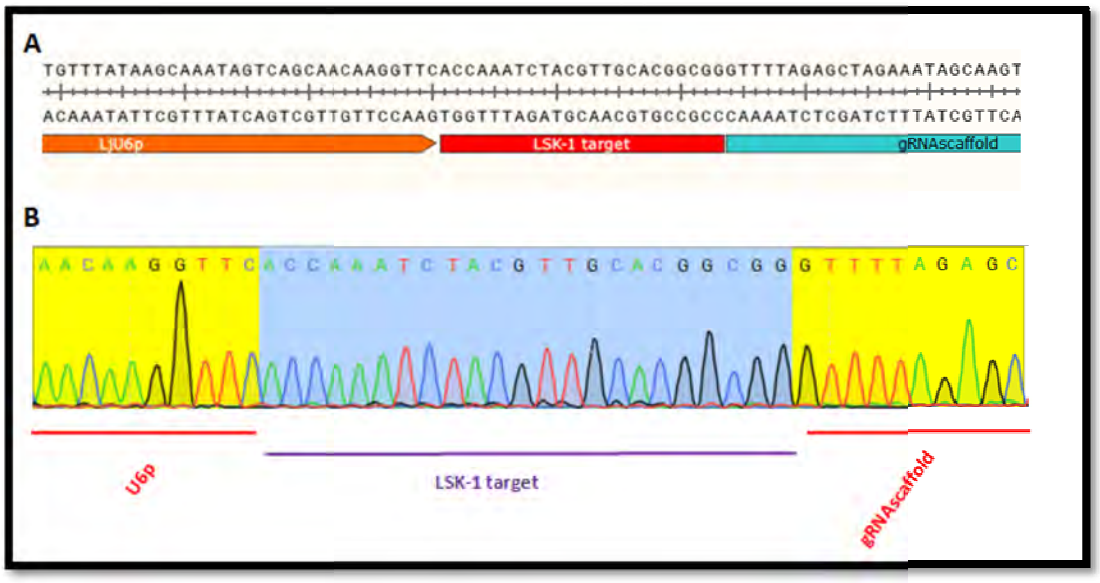

Figure43 gRNA:*LSK*-1 target vector A)*LSK*-1 target into gRNA_acceptor depiction B) sequencing trace of the gRNA:*LSK*-1 target vector

3.5 *Lotus japonicus* hairy root transformation for CRISPR/Cas9 system expression

pK7WGF2::hCas9 and gRNA:*LSK*-1target vectors were transformed into *Agrobacterium rhizogenes strain LBA9402* cells via freeze-thaw method (see Material and Methods) and cultivated into LB agar petri dishes with the appropriate selection for each one. These two transformed LBA9402 strains were utilized in order to express the CRISPR/Cas9 system into *Lotus japonicus* roots via hairy root transformation.

The primary root from 1-week-old *Lotus japonicus* seedlings was excised and the wounded hypocotyl region was inoculated with the *Agrobacterium* in order to develop hairy roots (see Hairy root transformation in Material and method). For the **expression of the CRISPR/Cas9 system**, we performed a co-transformation utilizing both the *Agrobecterium* strain transformed with the pK7WGF2::hCas9 and the one transformed with the gRNA:*LSK*-1target. As **controls** we used *Lj* seedlings transformed with *Agrobacteria* strains containing either Cas9 or gRNA:*LSK*-1target or gRNA_acceptor. In Table 3, the number of *Lotus japonicus* plants used for each Hairy Root transformation event is presented:

Table3 The four hairy root transformation events

Purpose	vector transformed in A.r. strain LBA9402	*Lj* plants
CRISPR/Cas9 expression	pK7WGF2::hCas9/ gRNA:*LSK*-1target	120
Control	pK7WGF2::hCas9	60
Control	gRNA:*LSK*-1target	60
Control	gRNA_acceptor	60

Fifteen days post inoculation with the *Agrobacterium*, the plants were transferred into sand-containing pots and then the plants were harvested for roots and shoots 29 days post inoculation. In Figure 44 the phenotypes of plants transformed with the Cas9/gRNA combination, Cas9 alone, gRNA:*LSK*-1target alone and an empty gRNA_acceptor vector are shown

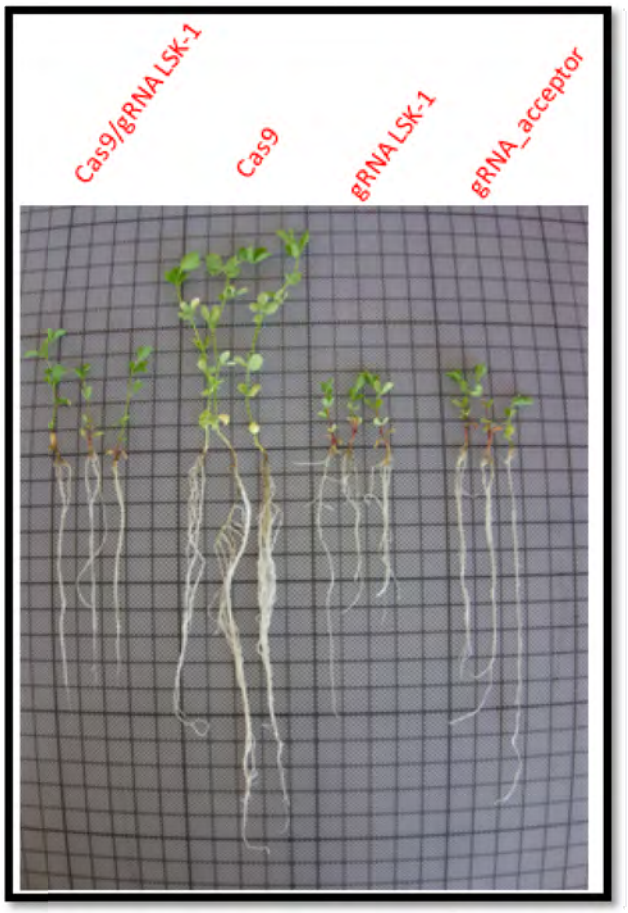

Figure 44 *Lotus japonicus* plants 29dpi

4. Conclusions/Discussion

As mentioned in the introduction, Legumes (plants of *Fabaceae or Leguminosae*family) possess a major nutritional value as components of the human diet in both developed and developing countries. In some regions of the world, legumes are the only protein source in people diet (Bouchenak and Lamri-Senhadji 2013). Furthermore, they play a major role in soil's nitrogen enrichment, as they have developed the capacity to establish symbiotic relationships with nitrogen-fixing rhizobacteria, developing new root organs called nodules (Oldroyd et al. 2011). Both legumes' nutritional and agricultural value, have received rising interest from researchers over the past decades. Among other legume model plants, *Lotus japonicus* is a model plant of choice for both classical and molecular analysis due to its favourable biological properties (diploid genome, self-fertile, susceptible to nudulation and mycorhizationetc) (J.Stuugard 2001).

In an effort to generate a genome editing tool compatible with *Lotus japonicus*, we optimised a CRISPR/Cas9 -based approach. Within this framework, we constructed one of the two basic CRISPR/Cas9 system components, a gRNA_acceptor vector, compatible with the *Lotus japonicus* background, and able to "host" and transcribe any gRNA designed to

target either *Lotus japonicus* genomic loci or phylogenetically related species because of the presence of the endogenous *Lotus japonicus* U6 promoter. In Figure 45, the procedures that were followed in order to obtain this gRNA_acceptor level 1 vector, are summarized.

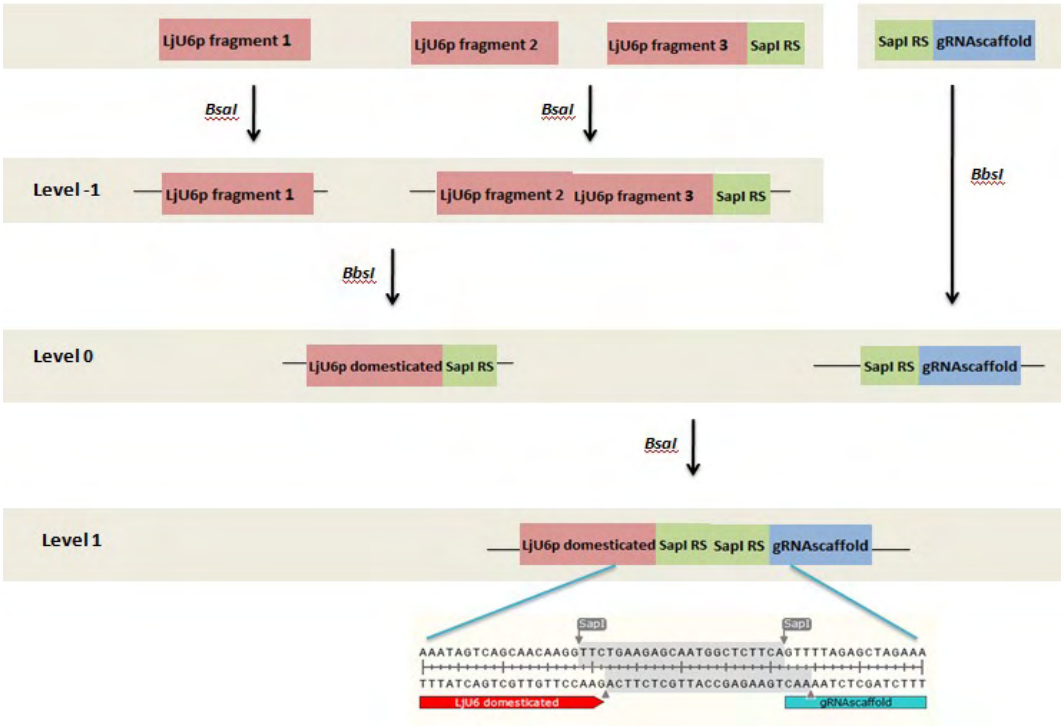

Figure45gRNA_acceptor construction. SapI RS: SapI Restriction site

Possessing such a binary vector that contains this expression cassette is a milestone for CRISPR/Cas9-mediated genome editing in *Lotus.* To be more specific, someone can utilize this binary vector in order to express any gRNA to target the desired genomic locus, only by performing a simple one-pot cloning reaction following the protocol we have standardized.

The gRNA_acceptor that we constructed may be an individual CRISPR/Cas9 component which could be utilized in *Agrobacterium*-mediated plant transformation, but it is also a synthetic biology's biopart. Thus, an expression cassette, a module in terms of Synthetic Biology that can be utilized, among others, for the construction of a bigger, much more complicated expression system. In more detail by combining several level 1 gRNA modules, each one expressing a different gRNA, one can develop a level 2 multi-gRNA binary vector that could express up to seven gRNAs. Concerning the significance of this vector in studies conducted *in planta*, we could concomitantly target up to seven different genomic regions without performing separate transformation events. A great example could be the concomitant mutagenesis of a group of genes that encode for protein-members of the same signalling pathway, and subsequently the elucidation of plant responses through the absence of this pathway.

The current thesis is a part of bigger project that is aiming in the construction of an all-in-one vector that can be utilized for CRISPR/Cas9-meciated genome editing in *Lotus japonicus* and related legume species such as *Trigonella*. Once again, in this project will be

performed the Synthetic Biology's core concept which is the arrangement of simple DNA-legos (bioparts) in order to construct a much bigger and complicated expression system containing up to seven bioparts. The bioparts needed for this construct are a Cas9 expression cassette, a selection marker or/and a reporter expression cassette and the gRNA expression cassette. We have already obtained the two out of three bioparts and the Cas9 biopart remains to be constructed. By following the same standardized cloning procedures performed for the gRNA_acceptor construction, we will be able to obtain the Cas9 coding sequence driven by the *Lotus japonicus* endogenous Ubiquitin promoter and followed by a *Lotus* terminator sequence. By accomplishing this final goal we will have the opportunity to increase both the system's transformation and *in planta* expression efficiency.

In order to test the newly-constructed gRNA_acceptor we proceeded to transient expression of the CRISPR/Cas9 system in *Lotus japonicus* roots, targeting a region lying at the 11th exon of *Lotus japonicus* Shaggy like kinase 1 (*LSK*-1). Proceeding to a stable transformation, we will have the opportunity to acquire a *LSK*-1-knocked out *Lotus* line which would be a fundamental tool for studying the Auto Regulation of Nodulation system in terms of plant responses in symbiosis with rhizobia. What remains to be done is the verification of *LSK-1* mutagenesis by PCR and sequencing analysis, and also the evaluation *LSK-1*expression via qRT-PCR.

To conclude, here we describe the construction of a valuable tool belonging to the new era of genome editing technologies in plants Biotechnology and Synthetic Biology. It will be of high significance in plant research not only for knocking out genes by developing random INDELs, like in the previous mentioned project targeting *LSK*-1, but also knocking them out by excising a whole DNA region by targeting flanking loci. These results cannot be obtained through the current transposons-mediated mutagenesis in higher plants, for that reason the "modern" CRISPR/Cas9 technology seems the ideal option.

5. References

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Figure 14*modified:Ferguson, Brett J., Dongxue Li, April H. Hastwell, Dugald E. Reid, Yupeng Li, Scott A. Jackson, and Peter M. Gresshoff. 2014. "The Soybean (Glycine Max) Nodulation-Suppressive CLE Peptide, GmRIC1, Functions Interspecifically in Common White Bean (Phaseolus Vulgaris), but Not in a Supernodulating Line Mutated in the Receptor PvNARK." Plant Biotechnology Journal 12(8):1085–97.

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